NEURONAL HYPOTHALAMIC PLASTICITY IN CHICKEN: A DEVELOPMENTAL AND PHARMACOLOGICAL PERSPECTIVE

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ABBREVIATIONS

PO/AH	preoptic area of anterior hypothalamus
ACSF	artificial cerebrospinal fluid
TC	temperature coefficient
GABA	gamma amino butyric acid
WS	warm sensitive
CS	cold sensitive
IS	Insensitive
Tb	body temperature
NTI	non-thermal inputs
CNS	central nervous system

SUMMARY

In the present electrophysiological studies, characterization of neuronal hypothalamic plasticity in the chicken brain slices aims to investigate the influence of age on thermosensitivity in the preoptic area of the anterior hypothalamus (PO/AH) by extracellular recordings in the age groups of 5, 10, 15, 20 and 30 days. Firing rate of neurons was recorded during sinusoidal temperature changes. Relationship between firing rate and temperature was used to calculate the temperature sensitivity of the neurons investigated. Investigations reveal high proportion of cold-sensitive neurons compared to the warm and insensitive neurons in some age groups. The high percentage of neuronal cold sensitivity has not been found in adult mammals and birds. In mammals and adult Pekin ducks the cold-sensitive neurons were less than 10 %. In chicken neuronal cold sensitivity shows an increase from day 5 until day 20 and is as high as 52 % in 20 days old birds. Between day 20 and 30 neuronal cold sensitivity again shows a major shift and declines to 34 %. Hence the high hypothalamic cold sensitivity seems to be a specific characteristic feature in juvenile birds. Between species a species specificity of the early development of neuronal hypothalamic thermosensitivity could be clearly demonstrated.

To study the inherent nature of cold-sensitive neurons synaptic blockade of cold-sensitive neurons were performed. These studies show the existence of inherent nature to a certain degree in the cold-sensitive neurons. It suggests a possible thermoregulatory role of cold-sensitive neurons in the chicken species in the observed age groups.

In the ensuing studies in the assessment of experimentally induced changes of neuronal temperature coefficients, emphasis was laid on the action of GABA in the local environment of the slice preparation. The effects of the GABAergic substances viz., $GABA_A$ receptor agonist muscimol, $GABA_A$ receptor antagonist bicuculline, $GABA_B$ receptor agonist baclofen and $GABA_B$ receptor antagonist CGP35348 on neuronal tonic activity (firing rate) and temperature sensitivity (temperature coefficient) of neurons in the PO/AH have been examined. Muscimol and baclofen in equimolar concentrations significantly inhibited the tonic activity of the hypothalamic neurons, regardless of their type of thermosensitivity. In contrast the $GABA_A$ receptor antagonist bicuculline as well as the $GABA_B$ receptor antagonist CGP 35348 increased the firing rate of the majority of the neurons. The temperature coefficient (TC) of chick hypothalamic neurons was significantly changed by ligands of $GABA_B$ receptors, and this effect was restricted to cold-sensitive neurons. The TC was significantly increased by $GABA_B$ receptor agonist baclofen and significantly decreased by the $GABA_B$ receptor antagonist CGP 35348. The

effects of muscimol and baclofen on firing rate and TC were prevented by co-perfusion of appropriate antagonists bicuculline and CGP 35348, respectively in tenfold higher concentration.

GABA mediated tonic inhibition resulting in modulation of firing rate and TC especially of cold-sensitive neurons possibly involved in the control of body temperature of the chicken represents 25.6 % of all neurons investigated in this part of the study. Thus the main effects of GABA, mediated via $GABA_A$ and $GABA_B$ receptors on thermosensitive and insensitive PO/AH neurons in the chicken are similar with that described in mammals. The only difference is in respect of the $GABA_B$ receptors mediated change in hypothalamic neuronal temperature sensitivity. In chicken this action was restricted to cold-sensitive neurons whereas in mammals this effect was only seen in warm-sensitive neurons. However, the results indicate that the fundamental mechanism of GABAergic influence on temperature sensitive and insensitive neurons in the chicken PO/AH are conserved during evolution. In the present investigations the responses of hypothalamic neurons to temperature changes suggest a possible functional role of GABAergic substances in the control of body temperature in birds.

Key words:

Chicken, Neuronal thermosensitivity, Brain slices, Fire rate, Pre-optical area of Hypothalamus anterior, young chicken, Temperature co-efficient, $GABA_A$ -Receptor-Agonist Muscimol, $GABA_A$ -Receptor-Antagonist Bicuculline, $GABA_B$ -Receptor-Agonist Baclofen, $GABA_B$ -Receptor-Antagonist CGP35348

ZUSAMMENFASSUNG

Eine Aufgabe der vorliegenden elektrophysiologischen Studie zur Charakterisierung der neuronalen hypothalamischen Plastizität beim Vogel (Haushuhn) bestand darin, den Einfluss des Alters (5., 10., 15., 20. und 30. Lebenstag) auf die neuronale Thermosensitivität der praeoptischen Region des *Hypothalamus anterior* (PO/AH) mittels extrazellulärer Ableitungen in Hirnschnitten zu untersuchen. Die neuronale Aktivität (Feuerrate) wurde während sinusförmiger Temperaturänderungen registriert. Aus der Beziehung zwischen Feuerrate und Temperatur konnte die Thermosensitivität der untersuchten Neurone bestimmt werden.

Im Vergleich zu wärmesensitiven und temperaturinsensitiven Neuronen wurde ein hoher Anteil kältesensitiver Neurone nachgewiesen. Eine derartig hohe neuronale Kältesensitivität wurde weder bei adulten Vögeln noch bei adulten Säugetieren beschrieben, bei denen der Anteil kältesensitiver Neurone weniger als 10% beträgt. Beim Hühnerküken erhöhte sich die neuronale Kältesensitivität vom 5. bis zum 20. Lebenstag und erreicht am 20. Lebenstag 52% der untersuchten Neurone. Zwischen dem 20. und 30. Lebenstag erfolgte wieder eine Abnahme des Anteils kältesensitiver Neurone an der Gesamtzahl aller untersuchten Neurone bis auf 34%. Die hohe neuronale Kältesensitivität scheint eine spezifische Eigenschaft des Hypothalamus juveniler Vögel zu sein. Die frühe Entwicklung der neuronalen hypothalamischen Thermosensitivität ist ausserdem deutlich artspezifisch.

Für den Nachweis inherent kältesensitiver Neurone wurden extrazelluläre Ableitungen unter synaptischer Blockade durchgeführt. Diese Studie ergab, dass einige Neurone eine inherente Kältesensitivität aufweisen. Eine mögliche zentrale Rolle kältesensitiver Neurone im Rahmen der Thermoregulation juveniler Hühner wurde postuliert.

Eine weitere Aufgabe der vorliegenden Arbeit bestand darin, experimentell durch GABA Applikation (Perfusion) den Temperaturkoeffizienten von PO/AH Neuronen zu beeinflussen. Der Einfluss GABAerger Substanzen, wie $GABA_A$-Rezeptor-Agonist Muscimol, $GABA_A$-Rezeptor-Antagonist Bicuculline, $GABA_B$-Rezeptor-Agonist Baclofen und $GABA_B$-Rezeptor-Antagonist CGP35348, wurde auf die tonische Aktivität (Feuerrate) und die Temperatursensitivität (thermischer Koeffizient) von PO/AH Neuronen untersucht. Muscimol- und Baclofenapplikation in gleichen Konzentrationen hemmen die tonische Aktivität der Hypothalamusneurone signifikant, unabhängig von der jeweiligen Thermosensitivität. Im Gegensatz dazu führen $GABA_A$-Rezeptor-Antagonist Bicuculline- und $GABA_B$-Rezeptor-Antagonist CGP35348-Gaben zu einem Anstieg der Feuerrate bei

der Mehrzahl der Neurone. Der Temperaturkoeffizient (TC) der Hypothalamusneurone beim Haushuhn wurde signifikant durch $GABA_B$-Rezeptor-Liganden verändert. Dieser Effekt beschränkte sich auf kältesensitive Neurone. Der TC wurde signifikant durch den $GABA_B$-Rezeptor-Agonisten Baclofen erhöht und durch den $GABA_B$-Rezeptor-Antagonisten CGP35348 gehemmt. Der Effekt von Muscimol und Baclofen auf die Feuerrate und den TC der Neurone wurde durch Co-Perfusion der entsprechenden Antagonisten Bicuculline und CGP35348 in einer 10-fach höheren Konzentration aufgehoben.

Die GABA induzierte tonische Hemmung führte hauptsächlich zu einer Modulation der Feuerrate und des TC kältesensitiver Neurone, die möglicherweise an der zentralen Kontrolle der Körpertemperatur beteiligt sind, und in diesem Teil der Studie 25,6% aller untersuchten Neurone betreffen. Insgesamt sind die wesentlichen Effekte von GABA, vermittelt über $GABA_A$- und $GABA_B$-Rezeptoren, auf thermosensitive und – insensitive Neurone mit den bei Säugetieren nachgewiesenen vergleichbar. Der einzige Unterschied bestand in der $GABA_B$-Rezeptor vermittelten Änderung der hypothalamischen neuronalen Thermosensitivität. Beim Hühnerküken betrafen diese Änderungen die kältesensitiven und beim Säugetier die wärmesensitiven Neurone. Die Ergebnisse deuten daraufhin, dass der grundlegende Mechanismus der GABAergen Beeinflussung thermosensitiver und – insensitiver PO/AH-Neurone einen älteren evolutionären Ursprung haben. Die aktuellen Untersuchungen lassen eine mögliche funktionelle Rolle GABAerger Substanzen im Rahmen der zentralen Kontrolle der Körpertemperatur beim Vogel vermuten.

Schlagwörtern

Haushuhn, Neuronale Thermosensitivität, Hirnschitte, Feurrate, der praeoptische Region des *Hypothalamus anterior*, juvenile Vögel, Temperaturkoeffizient, $GABA_A$ -Rezeptor-Agonist Muscimol, $GABA_A$ -Rezeptor-Antagonist Bicuculline, $GABA_B$ -Rezeptor-Agonist Baclofen, $GABA_B$ -Rezeptor-Antagonist CGP35348.

1 INTRODUCTION

1.1 Thermoregulation

1.1.1 Homeothermy: an evolutionary perspective

Continuous metabolically based maintenance of high and relatively stable body temperature in the face of the greatly fluctuating ambient temperatures is among the most remarkable attributes of mammals and birds. Such warm bloodedness namely homeothermy, generally results from a combination of high resting, aerobically supported heat production rates (much higher than reptiles) in virtually all soft tissues and insulation sufficient to retard excessive heat loss.

Similar to the mammals, the birds are homeothermic. Homeothermy is defined as "*The pattern of temperature regulation in a tachymetabolic species in which the cyclic variation in core temperature, either nychthermally or seasonally, is maintained with in the arbitrarily defined limits despite much larger variations in ambient temperature, i.e., homeotherms regulate their body temperature with in a narrow range*" (Glossary of Terms of Thermal Physiology, 2003).

Homeothermy is a characteristic feature of warm blooded animals. Chiefly, thermoregulation is done by endothermy, a mechanism of generating and maintaining internal body heat. In general this term is associated with mammals and birds.

Endothermy is defined as "*the pattern of thermoregulation in which the body temperature depends on a high (tachymetabolic) and controlled rate of heat production*" (Glossary of Terms of Thermal Physiology, 2003). Behavioural responses are often used by endotherms.

Reptiles, from which birds have evolved (Baker 1975) and Archeopteryx being the missing link are poikilothermic. Poikilothermy in the Glossary of Terms of Thermal Physiology (2003) has been defined as a "*large variability of body temperature as a function of ambient temperature in organisms without effective autonomic temperature regulation*".

Endogenous heat production is insufficient to alter the body temperature and internal heat is necessarily derived primarily from the environment and this could be termed as ectothermic poikilothermy.

Ectothermy is defined as "*the pattern of temperature regulation of animals in which body temperature depends mainly on the behaviourally controlled exchange of heat with the environment*" (Glossary of Terms of Thermal Physiology, 2003). Autonomic

thermoeffectors may be temporarily important in a few ectothermic species (panting in lizards, warming up of insects).

As in endotherms, the reptilian hypothalamus is primarily thermostatic control centre and many lizards thermoregulate behaviourally during extended periods of diurnal activity at body temperatures that overlap those of endotherms. Mammals, birds and also of extant reptiles exhibit an adaptive endotherm like response in which the thermoregulatory set point is elevated significantly by the action of endogenous and exogenous pyrogens on the hypothalamus. Accordingly attainment of avian or mammalian metabolic status need not have involved radical modification of ancestral thermostatic sensors or temperature regulatory regimes maintained behaviourally by the reptilian progenitors of this group.

The evolution of homeothermy has brought about a highly advantageous increase in the adaptability of changing environments. A constant body core temperature optimises physiological processes and increases the ability of an individual to sustain activity, growth rate and reproduction rate when exposed to low ambient temperatures.

By using avian development as a model system for transition from ectothermy to endothermy, Shabtay and Arad (2005) show that, in contrast to the ectothermic state, in the endothermic state the organism is more resistant to heat but relies less on heat shock proteins (HSPs) as a first-line thermoprotective mechanism. Moreover, intraspecific, real-time, *in vivo* measurements in genetically diverse fowl strains relate improvement of thermoresistance in endotherms to improved body temperature (Tb) regulation, with a concomitant delay in the expression of HSPs. The time course of this delay and the Tb at which it occurs imply that the ontogenetic and evolutionary pathways leading to improved thermoresistance may have followed two, apparently non-related, parallel routes – cellular and peripheral (noncellular). In search of other cellular components that differentially participate in the heat shock response, a significant expression of fatty acid synthase (FAS) in heat-exposed endotherms was revealed but not in ectotherms**.**

Most animals use aerobically based metabolism to fuel low to moderate levels of activity. Endothermic metabolic physiology is ultimately associated with profound restructuring at the molecular, cellular and organ system levels of organisation (e.g., increased levels of membrane polyunsaturated phospholipids, high power muscle mitochondria, elevated tissue protein and phospholipids content, pulmonary and cardiovascular specialisations, etc) (Ruben 1995).

In mammals the exercise metabolism is 10 times higher than in the reptiles of the same body size and at a similar body temperature, and consequently their basal metabolism is also 10 times that of the comparable reptiles. In birds exercise and basal metabolism are even higher than in mammals, with a characteristic differentiation between non-passerine and passerine birds, the latter showing the highest metabolic rates (Gordon *et al.,* 1982; Wieser 1986). These system characteristics are the reason for the height of the basal metabolic rate in homeotherms. All other relationships are consequences of inherent functional interdependencies. The drastic elevation of the basal metabolic rate in the developing birds and mammals with their continuous high rate of metabolic heat production enabled these vertebrates to couple this ability with a continuously functioning thermoregulation to maintain a constant body temperature and to regulate it by slight changes in the basal metabolic rate. In this way the homeothermy resulted as a final step in the specific coupling of the evolved locomotor, cardiovascular, respiratory and cellular metabolic systems (Duncker 1991). These systems possess as a consequence multiple interrelationships, such as an advanced thermoregulation supported by the development of insulating structures and mechanisms such as feathers or hairs. Homeothermy could not have been selected stepwise in phylogeny as the evolutionary development of the aerobic capacity, but emerges as a new functional quality as a consequence of the functional integration of these highly evolved systems. Once developed, homeothermy provided birds and mammals with new biological and ecological possibilities. (Duncker 1991).

Advantages of homeothermy in early ontogeny

There are advantages to homeothermy and thermoregulation in the early post hatch period. In precocial species, the development of these capabilities at early post hatch ages allows independent locomotion and foraging for food (McNabb and Olson 1996).Thermogenic capacity increases steadily during the post hatch period as the chicks acquire greater metabolic capacity, a more favourable surface to volume ratio and increased insulation is facilitated. Parental brooding can provide heat to the chicks during environmental conditions more severe than those at which the chick can maintain homeothermy. In contrast to this relatively self-sufficient precocial pattern, altricial young initially are essentially poikilothermic and do not respond thermogenically to cooling. Altricial chicks are physically relatively helpless and therefore dependent on parental care for food, protection and heat. In general, the lack of energy expenditure for thermogenesis during the early post hatching period appears to be the key to the rapid body growth during this time in most altricial species (McNabb and Olson 1996). This differs from the slower

growth of precocials, whose energy supply is used for thermogenesis and locomotion and as well as for growth (Vleck and Vleck, 1987). In contrast to these differences in patterns of heat production, heat dissipating mechanisms are present at hatching both in precocial and altricial chicks. Compared to physiological regulation of body temperature, behavior is a phylogenentically older but very effective means of thermoregulation (Kluger 1979). In the early postnatal phase thermoregulatory system matures. During this period behavioral thermoregulatory mechanisms, such as the innate ability to prefer ambient temperature are essential for maintenance of homeothermy because the autonomic mechanisms of thermoregulation are not fully developed (Tzschentke and Nichelmann 1999).

In nature, groups of chicks huddled together in protected situations such as nests, may maintain constant body temperatures (effective homeothermy) at earlier ages than those when they are capable of appreciable endothermy. This may indicate that neural sensory control is mature by hatch or earlier in all avian young, but that only the effectors for heat production are delayed in their maturation in altricial species (McNabb and Olson 1996).

1.1.2 Operation of thermoregulatory mechanisms

1.1.2.1 Basic mechanisms of the regulatory system

The stability of deep body temperature is the result of action of a regulatory negative feedback system, in which the controlled variable is an evaluated body temperature. Several parts of the body possess receptors which transduce temperature to nervous activity and in accordance to their relative thermoregulatory importance (Simon *et al.,* 1986), induce autonomic responses such as non-shivering thermogenesis, vasomotor control of skin blood flow, piloerection, and sweating, panting, as well as behavioral responses via diverse efferent pathways (Gilbert and Blatteis 1977; Asami *et al.,* 1988). Besides cutaneous cold- and warm-receptors, thermopreceptors have been found subcutaneous (Ivanov *et al.,* 1982), in muscles (Jessen 1985), the dorsal wall of abdominal cavity (Simon 1974) as well as with in the spinal cord and lower brain stem (Jessen 1985). Afferent transmission of temperature signals is mediated via spinothalamic tracts or multisynaptic brain stem pathways to the hypothalamus (Brück and Hinckel 1982). Facial temperature signals are well represented in the caudal trigeminal nucleus (Dostrovsky and Hekkon 1978) from where they ascend to the hypothalamus.

Feedback system

This process could be summarized in a feed back mechanism as represented in figure 1. The main elements of the feedback system constitute receptors, effectors, controlled variable, central 'thermostat' and error signal. The deep body temperature as a controlled variable is measured by means thermistor probe. At the same time the external stimuli (like environmental factors) all arraign to a setpoint signal where in all these aspects act instinctly via the error signal with their action on the effectors which results in endocrine and autonomous functions inducing certain behavioral changes as well.

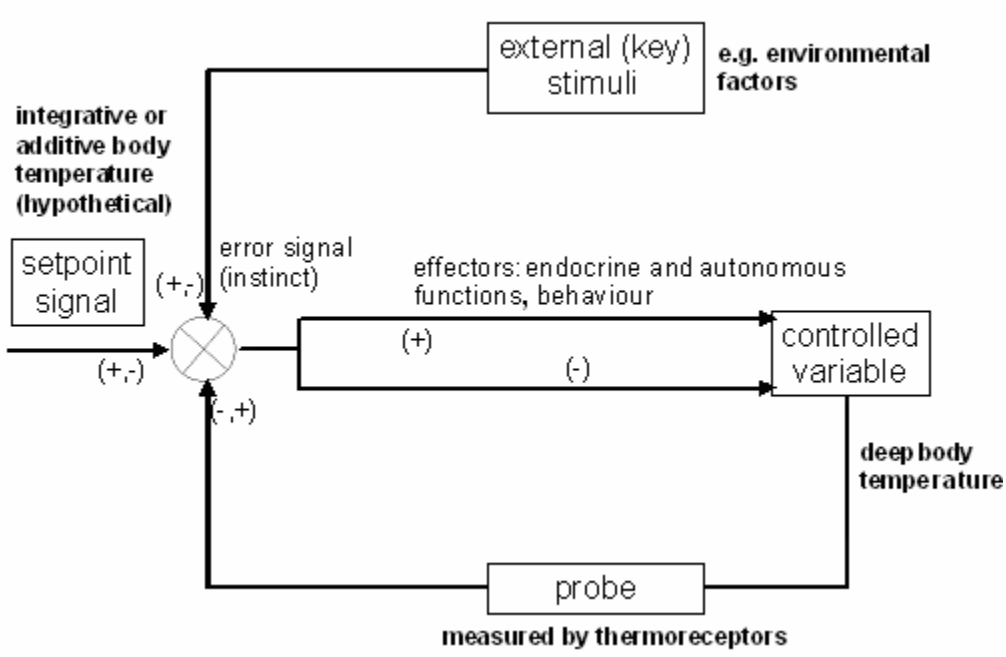

Figure 1: Elements of feedback system.

During this process different factors viz., the external stimuli like the environmental factors reach a set point signal. It occurs as a consequence of multiple factors acting on the controlled variable which involves the effectors with the endocrine and autonomous functions facilitating the thermoregulatory action affecting the deep body temperature. This deep body temperature could be measured by inserting thermistor probes which could be ascertained at the set point of a hypothetical situation of integrative (additive) body temperature.

1.1.2.2 Neuronal mechanisms

1.1.2.2.1 Role of preoptic area of anterior hypothalamus (PO/AH)

It has been clearly identified by both *in vitro* and *in vivo* models that preoptic area of anterior hypothalamus **(**PO/AH) plays a pivotal role in thermoregulation. The preoptic area and suprachisamatic portion of the anterior hypothalamus (PO/AH) is considered to be a primary region where central and peripheral thermal information is integrated to produce appropriate thermoregulatory responses (Boulant 1980).

Early studies in mammalian thermoregulation showed that, thermal stimulation of the hypothalamus and the preoptic area evoked heat loss responses through local warming (Magoun *et al.,* 1938) as well as heat production/heat retention responses through mild local cooling respectively (Freeman and Davis 1959; Hammel *et al.,* 1960, 1963; Banet *et al.,* 1978). Further, thermal stimulation of PO/AH produces appropriate behavioural thermoregulation (Adair 1974; Satinoff and Hendersen 1977).

1.1.2.2.2 Paradoxical and inappropriate reactions of thermoregulatory responses in birds

Birds unlike most mammals, display interspecific differences in their thermoregulatory response to changes in PO/AH. However appropriate heat-loss responses in most of the avian species investigated so far, heat loss and heat retention responses often appear either weak or inappropriate during PO/AH cooling.

In the domestic fowl (Scott and van Tienhoven 1974) and Californian quail (Snapp *et al.*, 1977) cooling of PO/AH induces no changes in temperature regulation. In ducks (Simon-Oppermann *et al.,* 1978), penguins (Simon *et al.,* 1976) and pigeons (Rautenberg *et al.,* 1972), a local cooling inhibits shivering and cutaneous vasoconstriction at low ambient temperatures, where as it induces panting at high ambient temperatures. In other avian studies the hypothalamus appears to be highly thermosensitive and responds to temperature in a manner similar to that observed in mammals. Thermal stimulation of the rostral brain stem induces appropriate heat loss and heat production in emu (Jessen *et al.,* 1982), goose (Helfmann *et al.*, 1981) and house sparrow (Mills and Heath 1972).

The differences in avian thermoreception appear to exist only for autonomic, and not for behavioural responses. The rostral hypothalamus still remains highly thermosensitive in terms of controlling behavioural thermoregulatory responses (Schmidt 1976; Schmidt and Simon 1979). Hence behavioural and autonomic thermoregulatory neuronal networks may be functionally and anatomically distinct. Moreover paradoxical

and inappropriate reactions of thermoregulatory responses might also be associated with an unphysiological level of hypothalamic cooling of upto10 °C.

Some possible explanations have been extended to explain weak or partially paradox responses of thermoregulatory effectors to hypothalamic thermal stimulation in some species of birds as opposed to mammals. The thermoregulatory system in homeothermic organisms has been characterised as a "multiple input system" (Brück and Wünnenberg 1967; Simon 1974). In this the controlled variable is the "setpoint temperature", a constant average body temperature rather than a constant temperature in a limited area of body core (Brück and Hinke1 982). In Pekin ducks, the relationship between the efficiency of whole body cooling and selective hypothalamic cooling in stimulating metabolic cold defence showed that hypothalamic cooling is quantitatively irrelevant compared with whole body responsiveness (Simon 1974). This conforms to the hypothesis that, the distribution of central nervous sensitivity might have followed different lines of evolution in mammals and birds and could explain why metabolic cold defence in some species of birds is not inferior to that of mammals in natural conditions of cold stress (Simon *et al.*, 1986). In some avian species thermosensitive neurons controlling autonomic thermoregulation may be located in the lower central nervous system, such as in the brain stem or spinal cord (Simon 1974), in order to be more representative for body temperature.

1.1.2.2.3 Neuronal model

Thermal stimulation studies of discrete brain locations in mammals, which induce autonomic (Magoun 1938; Hammel *et al.,* 1963) and behavioural (Satinoff and Rutstein 1970; Cabanac and Bassam 1983) thermoregulatory responses, have been the basis for the concept that the hypothalamus also contains central thermoreceptors itself and functions as a "central thermostat" and is a major tenet of thermoregulatory physiology (Boulant *et al.,* 1989). Integration of afferent inputs within the hypothalamus and elicitation of adequate thermoregulatory responses to keep a stable deep body temperature has been first described through a neuronal model developed by Hammel (1968) and further adpated Nichelmann (1994) in figure 2.

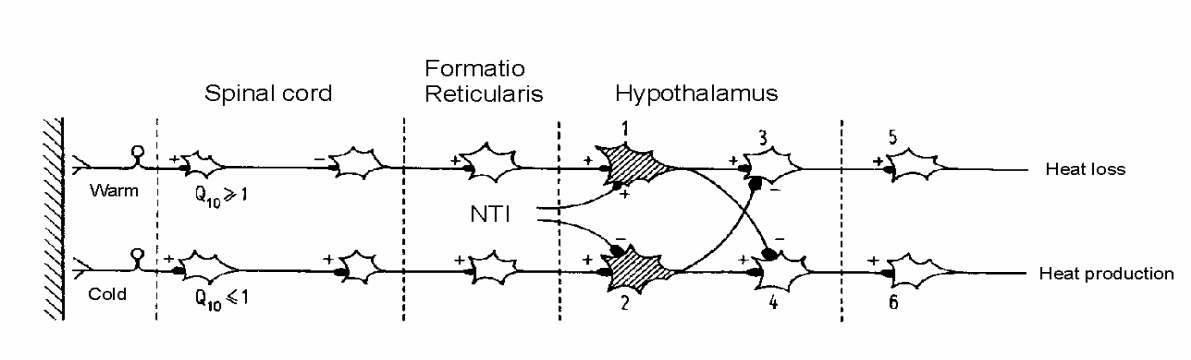

Figure 2: Model of thermoregulatory integration by Hammel (1968) and -further adapted by Nichelmann (1994). (NTI = non thermal inputs).

This model suggests that the ascending pathway through the brain stem (*Formatio reticularis*) brings somatosensory information from the skin, extracerebral core and spinal thermoreceptors to the hypothalamus. Warm-sensitive neurons of type **1** are inherently thermosensitive where as cold-sensitive neurons of type **2** are merely temperature insensitive neurons receiving inhibitory synapses from near by warm-sensitive neurons. PO/AH warming would increase the synaptic inhibition and cold-sensitive neurons would increase their firing rate. Depending on afferent inputs of neurons type **1** and **2**, as well as through the reaction of neurons type **1** on hypothalamic temperature, interneurons of type **3** and **4** would be stimulated or inhibited respectively. Although insensitive to temperature changes themselves they receive excitatory and inhibitory inputs from extracerebral as well as central nervous temperature afferents. In case that excitatory input on one type of neuron are high enough, adequate heat retention or heat producing responses are elicited via excitatory signal to motor neurons (type **5** or **6**). In addition non-thermal inputs (NTI) i.e., local osmotic pressure (Boulant and Silva 1988), can further modulate thermoregulatory mechanisms. In this model afferent inputs of all thermoreceptors within the body have an influence on heat production, although the inner thermoreceptors seem to have a strong influence as it is of major importance for homeothermic animals to keep their deep body temperature at a constant level (Simon *et al.,* 1986).

The posterior hypothalamus coordinates heat production and retention mechanisms as a response to cold stress and serves as a motor function in shivering (Hardy 1973; Boulant 1980) where shivering is induced by electrical stimulation (Stuard *et al.*, 1961) and abolished by lesions (Stuard *et al.,* 1962). Electrical stimulation of the ventromedial hypothalamus in anaesthetised rats specifically activates non-shivering (brown adipose tissue) effector mechanisms to raise core temperature (Thornhill 1994). This hypothesis of regional differences in the control of shivering and non shivering thermogenesis was

further supported by investigations in guinea pigs (Brück and Wünnenberg 1970). Moderate PO/AH cooling in these animals elicit nonshivering thermogenesis, not shivering. Moderate cervical spinal cord cooling, however will elicit shivering. The lateral hypothalamus plays a crucial role in behavioural thermoregulation (Satinoff and Shan 1971) and the control of circadian body temperature rhythms (Fischette *et al.*, 1981). It is also permeated by a large fiber tract, the median fore brain bundle, which interconnects all hypothalamic nuclei (Nieuwenhuys *et al.*, 1984), suggesting that this area is an integrative region where thermal pathways converge.

Furthermore there seem to be thermosensitive areas in the lower brain stem and spinal cord. Local warming of the medulla evoked heat retention mechanisms in the rabbit (Chai and Lin, 1973). Recent electrophysiological analysis has identified some neurons sending axons directly to the spinal cord for thermoregulatory effector control. Included are midbrain reticulospinal neurons for shivering and premotor neurons in the medulla oblongata for skin vasomotor control (Nagashima *et al.,* 2000).

1.1.2.2.4 Characterization of thermosensitive neurons in the hypothalamus

The PO/AH contains thermosensitive neurons that respond to small changes in local hypothalamic temperature by a change in the firing rate. Firing rate or the frequency of action potentials is generally measured as impulses per second. Cells that increase their firing rate with increases in local brain temperature are classified as warm-sensitive and are thought to stimulate heat loss and inhibit heat gain mechanisms. The converse is true for cold-sensitive neurons (Boulant and Hardy 1974).

Since the first reports (Nakayama *et al.,* 1961) on single-unit recordings of thermosensitive neurons, such neurons have been found in a wide species of homeothermic (Simon *et al.*, 1977; Hori and Shinohara 1979; Nakashima *et al.,* 1987) and poikilothermic (Cabanac *et al.,* 1967; Greer and Gardner, 1970; Nelson and Prosser 1981) vertebrates, studied both *in vivo* and *in vitro* (Hori 1981; Scott and Boulant 1984). Although found in different areas within the brain (Vasilenko and Gourine 1992) most of these electrophysiological single unit studies have focussed on the PO/AH and indicated that approximately 20-30% of neurons are warm-sensitive, 5-10% are cold-sensitive and 60-70% are temperature insensitive (Boulant 1980; Hensel 1981) in all vertebrate species investigated. Warm and cold responsive neurons occur in the PO/AH region of adult birds with similar frequencies as in mammals, as demonstrated with the Pekin duck both *in vivo* (Simon *et al.,* 1977) and *in vitro* (Nakashima *et al*., 1987). In contrast to results obtained in adult Pekin ducks (6.2% cold sensitive neurons, Nakasimha *et al.,* 1987) and mammals

(cold sensitive neurons <10%; Boulant and Dean 1986), in embryos and in 1-to 5-day old Muscovy ducklings the percentage of cold sensitive PO/AH neurons was much higher (up to 30%, Tzschentke and Basta 2000). This high hypothalamic cold sensitivity suggests a possible thermoregulatory role of cold-sensitive PO/AH neurons in prenatal and juvenile birds.

Definition of neuronal thermosensitivity

Investigations on thermosensitivity have used as criteria either the slope of the thermoresponse curve (temperature coefficient (TC) in imp/s/°C) or the Q_{10} of the firing rate. The Glossary of Thermal Physiology (2003) defines the Q_{10} as "*the ratio of the rate of the physiological process at a particular temperature to the rate at a temperature of 10°C lower, when the logarithm of the rate is an approximately linear function of temperature*". Neurons whose firing rates double (over a "theoretical" 10°C range) have a Q_{10} value of 2, the Q_{10} is less than 0.5 if the firing rate decreases by one half. Slope is preferred in some studies because it can be applied to both, the local thermosensitivity of the neuron as well as peripheral thermosensitivity due to afferent input from cutaneous thermoreceptors (Boulant and Hardy 1974).

Thermosensitive neurons are generally defined as the neurons which respond to local temperatures in the range between 35 and 42 °C with Q_{10} over 2 and/or TC of greater than or equal to 0.6 imp/s/°C (warm-sensitive) and with Q_{10} over 2 and/or TC of smaller than or equal to -0.6 imp/s/°C (cold-sensitive) over a temperature range of minimum 2 °C temperature. According to this definition all other cells are regarded as temperature insensitive (Nakashima *et al.,* 1987). The applied current method for the classification of single neuron thermosensitivty is the limitation value of TC.

The present method disregards thermosensitive neurons like temperature guardian neurons (Basta *et al.,* 1997), which exclusively react to signal critical brain temperatures around 36 or 41 °C in a relatively small temperature range around 0.2 to 0.6 °C and also threshold temperature neuorns (Vasilenko and Gourine 1992). They may possibly stimulate a second type of thermoregulatory effector activity, activated only when the body temperature deviates markedly from the normal range (Bligh 1966).

Neuronal differences in thermosensitive units

To study the neuronal differences, tissue slice and tissue culture studies have used high magnesium-low calcium perfusion to determine the effect of chemical synaptic blockage

on individual thermosensitive neurons (Hori *et al.,* 1980; Baldino and Geller 1982). Neurons that retain their thermosensitivity during synaptic blockage (inherently thermosensitive neurons) may function as central thermodetectors. Thermosensitive neurons that become temperature insensitive during blockage (conditionally thermosensitive neurons) may function as interneurons in thermoregulatory networks. The presence of central warm and cold thermodetectors, as well as various interneuronal cell types have been hypothesised (Bligh 1979). Inherently warm as well as cold-sensitive neurons could be investigated in most areas of the diencephalons (Dean and Boulant 1989). Other investigators suggest that only warm thermodetectors exist, from which all other neuronal responses are derived through synaptic interactions (Boulant and Hardy 1974).

Earlier *in vivo* studies (Hellon 1967; Guieu and Hardy 1970; Boulant 1974) revealed that the character of temperature activity relationship is different in different thermosensitive neurons. In some cells the firing rate depends on a wide range of temperature, while other cells are excited only at a certain threshold temperature close to normal brain temperature (Vasilenko and Gourine 1992) or critical brain temperature (Basta *et al.,* 1997 and Tzschentke and Basta 2000). Such differences are thought to reflect connections of different thermosensitive neurons with different effector thermoregulatory processes (Boulant 1974; Boulant *et al.,* 1989). The hypothesis that physiological differences between temperature insensitive and temperature sensitive PO/AH neurons were mirrored in their morphological differences, has shown in studies that allowed identified neurons to be labelled with an intracellular dye (Griffin and Boulant 1991).

Multimodal responses of preoptic and anterior hypothalamic neurons

A high degree of convergence of thermal and non-thermal homeostatic signals of the PO/AH neurons together with abundant neural connections between PO/AH and divergent areas of the brain suggests that PO/AH thermosensitive neurons may be involved in the coordination of thermoregulation and nonthermal autonomic and behavioural responses controlled by the hypothalamus (Hori *et al.,* 1987). 40 to 70 % of PO/AH thermosensitive neurons respond to non thermal homeostatic parameters such as local osmotic pressure, blood pressure, glucose, testosterone, estradiol (Boulant and Silva 1988) and non thermal emotional stimuli (Hori *et al.*, 1987). Daily changes in body temperature sensed by thermosensitive neurons within the central nervous system may help synchronise the circadian clock (Burgoon and Boulant 2001). This suggests that, even at the neuronal level,

there is a basis for interactions between homeostatic systems (Hori *et al*., 1987 and Boulant and Silva 1988).

1.1.2.2.5 Important conditions for comparison between different investigations

The limitation value of TC facilitates the comparison between different investigations on neuronal hypothalamic thermosensitivity. This enables comparison between *in vivo* and *in vitro* investigations on thermosensitive units within different brain areas or within different animal species.

Apart from the limitation value of TC, a temperature stimulus within precisely the same temperature range throughout different experiments and rapid, exact temperature changes without any overshoot of controlled temperature are required. Investigations on neuronal hypothalamic thermosensitivity (Schenda 1993; Basta 1995) indicate that the determined temperature sensitivity of a neuron extremely depends on the investigated temperature range. Because of the non-linear firing characteristics in most neurons a change of temperature range in which the experiments are carried out may cause changes in recorded temperature sensitivity of single neurons. According to Basta (1995), a shift of 1°C in the investigated temperature range to lower or upper temperatures may result in an apparent transformation of former temperature sensitive neurons into insensitive ones or opposite, because another part of the exponential function which describes the temperature dependent firing rate over a wide temperature range is recorded.

Finally basic temperature before temperature stimulus and temperature changes within a natural physiological range are of major importance. During sensory and motor activation and development of hyper- or hypothermia, brain temperatures in homeothermic animals deviates from the normal levels not more than 2-3°C, at a rate as a rule, not over 0.2°C/minute (Abrams and Hammel 1964; Meisenberg and Simmons 1984; Kruk *et al.,* 1985).

1.1.2.2.6 Cellular Mechanisms of thermosensitivity

Warm-sensitive neurons

Neuronal warm sensitivity might occur as a result of passive depolarisation associated with the effect of temperature on the ratio of Na^+- and K^+- permeability (Carpenter 1967; 1970). This implies that in warm-sensitive neurons the thermal effect may be greater on sodium permeability (P_{Na}) than Potassium-permeability (P_K). Therefore at higher temperatures there would be relatively more depolarising Na^+-current compared to the hyperpolarising K^+-current. This depolarisation would result in an increased firing rate with warming.

This hypothesis is challenged by intracellular recording studies that indicate that firing rate temperature sensitivity is not due to thermally dependent changes in the resting membrane potential, action potential threshold, or amplitude of the fast after-hyperpolarising potential. Instead these studies suggest that the primary mechanism of neuronal thermosensitivity resides in the depolarising potential, which is the slow depolarisation that occurs prior to the membrane potential reaching threshold (Burgoon and Boulant 2001). In warm-sensitive neurons, warming causes an increase in the rate of rise in the depolarising prepotential that precedes each action potential, such that threshold is reached sooner. This shortens the interspike interval between consecutive action potentials and increases the firing rate (Curras *et al.,* 1991; Boulant 1995; Griffin *et al.*, 1996; Burgoon and Boulant 2001).

Cold-sensitive neurons

The cellular basis of neuronal cold sensitivity remains controversial. Initial studies of neuronal cold sensitivity have concentrated on thermal effects on the electrogenic Na^+/K^+ pump (Senf 1967; Carpenter 1970, 1973). This pump transports sodium ions out of cells and potassium ions into cells. Since the pump is metabolically driven it can be accelerated with warming and slowed with cooling and has been found to contribute significantly to the resting membrane potential. An increase in pump activity tends to hyperpolarise neurons, which may be associated with decreased firing rates. Conversely cooling would tend to depolarise neurons which may be associated with increased firing rates. Applications of oubain, which blocks the Na^+/K^+ pump, either blocks or reduces cold sensitivity (Spray 1974; Pierau *et al.,* 1975).

However, the lack of oubain effect on thermosensitivity in other investigations suggests that cold sensitivity might not be due to thermal effects on the Na^+/K^+ pump (Curras *et al.,* 1986). An alternative hypothesis for cold sensitivity states that in some cells potassium permeability (P_k) is much more thermally dependent than sodium permeability (P_{Na}) (Klee *et al.,* 1974; Pierau *et al.*, 1976). Therefore cooling markedly reduces potassium permeability, resulting in depolarisation and an increased firing rate.

Other investigations support the neuronal model by Hammel (1965) and Boulant (1985). As mentioned previously they proposed that cold-sensitive neurons are not inherent but could be rather viewed as interneurons receiving inhibitory synaptic input by the near by warm-sensitive neurons. It is this inhibition that imparts the apparent cold sensitivity in these neurons. Intracellular studies of PO/AH neurons (Nelson and Prosser 1981;

Perlmutter and Boulant 1983) support this view. In both studies the thermosensitivity of cold sensitivity of neurons appear to be highly dependent on excitatory and inhibitory post-synaptic potentials, which implies that cold-sensitive neurons are synaptically driven by other neurons nearby. Further evidence for this hypothesis comes from several different electrophysiological studies, including tissue slice studies, where recorded cold sensitivity is often lost during perfusions with high magnesium-low calcium media that reversibly blocks synaptic transmission (Kelso and Boulant 1982; Dean and Boulant 1989)

In contrast to this within other experiments cold-sensitive neurons tested in synaptically blocking medium did not change, both, firing rate as well as their cold-sensitivity during synaptic blockage, thus showing inherent cold sensitivity (Hori *et al.,* 1980; Nakashima *et al.,* 1987).

Temperature insensitive neurons

Temperature sensitive neurons may be envisioned as having two opposing mechanisms: a thermally sensitive P_{Na}/P_K ratio (similar to warm-sensitive neurons) and a significant electrogenic pump activity associated with the thermosensitivity of certain cold-sensitive neurons. When summated these two mechanisms may render the neuron thermally insensitive. The only supporting evidence for this hypothesis comes from a study from which neurons were recorded from rat PO/AH tissue slices before, during, and after oubain perfusion (Curras *et al.*, 1986). When the Na^{++}/K^{+} pump was blocked, approximately 40 % of the temperature insensitive neurons displayed warm sensitivity. This suggests that a hyperpolarising effect of the Na^{++}/K^{+} pump may counteract the depolarising effect of the Na^{++}/K^{+} ratio which would allow the firing rate of certain neurons to be unaffected by temperature changes (Boulant *et al.*, 1989). In addition intracellular recordings on hypothalamic neurons revealed that temperature stimulation had little or no effect on the prepotential rates of depolarisation in temperature insensitive neurons (Boulant 1995; Burgoon and Boulant 2001).

Related cellular mechanisms

The molecular and cellular mechanisms of thermosensitivity in central neurons are not yet understood clearly. In a recent study on PO/AH neurons in culture, Tabarean (2005) have shown that the endogenous pyrogen, prostaglandin E2, can unmask the thermosensitivity of “temperature-insensitive” neurons by lowering the inhibition they receive, through a presynaptic mechanism which involves the inhibition of the extracellular signal-regulated

kinase (Tabarean 2005). This finding suggests that the degree of neuronal thermosensitivity is modulated by synaptic activity and that it is a more adaptive property than previously thought. The results from the present work carried out in cultured embryonic neurons were not substantially different from those obtained by other authors in studies carried out in PO/AH slices (Curras *et al.,* 1991; Kobayashi and Takahashi, 1993; Griffin *et al.,* 1996, 2001) or cultured PO/AH explants (Baldino and Geller, 1982).

One hypothesis suggests that temperature could affect steady state currents that determine the resting membrane potential, resulting in an increasing firing rate (Kiyohara *et al.*, 1990; Kobayashi and Takahashi 1993).

Recent studies suggest that most PO/AH neurons have the same types of ionic channels, but different levels of channel expression can explain the inherent properties of the various types of temperature sensitive and insensitive neurons. Both warm-sensitive and temperature-insensitive neurons displayed excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs). In most cases, EPSP and IPSP frequencies were not affected by temperature changes, suggesting that temperature insensitive neurons are responsible for most local synapses within this hypothalamic network (Griffin *et al.,* 2001).

Neuronal thermosensitivity and TRPV channels

Recent studies propose that neuronal thermosensitivity is due to thermally induced changes in persistent, inward, cationic currents that determine the resting membrane potential. Heat-sensitive and vanilloid/capsaicin-sensitive transient receptor potential vanilloid (TRPV) channels have been identified in the dorsal root ganglion and are suggested to be transducers of hypothalamic temperature sensitivity (Caterina *et al.,* 1997; Guler *et al.,* 2002; Benham *et al.*, 2003; Patapoutian *et al.,* 2003). These calcium and sodium TRP channels produce warm-induced depolarization which could produce increased firing rates. Studies by Kiyohara et al. (1990) and Kobayashi & Takahashi (1993) indicate that PO/AH neuronal thermosensitivity is due to a warm-induced membrane depolarization caused by a non-inactivating, inward sodium current.

The vanilloid derivative capsaicin, is the pungent ingredient in red peppers of the genus Capsicum including chilli and the sensory experience associated with its intake ranges from pleasant to painful nature. However, the great divide between pleasant and repellant sensations is rooted much deeper within the vertebrate kingdom. Birds are not repelled by capsaicin at all, because the avian ortholog of the transient receptor potential

(TRP) ion channel of the vanilloid type 1 (TRPV 1), which represents the bcapsaicin receptor Q in mammals, lacks the vanilloid binding site (Jordt and Julius, 2002). Hence the basic mechanism of thermoregulation between the mammals and birds might have similarities but there might exist specific differences in relation to thermoregulation in birds as they are devoid of the vanilloid binding site.

1.1.3 Early ontogeny of thermoregulation in birds

Prenatal development of peripheral thermoregulatory mechanisms

First rhythmic contractions of the respiratory muscles start piercing the chorio-allantoic and internal egg membrane (internal pipping). These movements are without ventilation of the lung. One goal of this movement is to consolidate the morphology and function of the respiratory tract (Tazawa, 1987; Murzenok *et al.,* 1997). In Muscovy duck embryos between internal and external pipping panting reactions were found when body core temperature increased. Like in adult birds, two phases of panting occurred in Muscovy duck embryos also. It was also observed that, after internal pipping in bird embryos, blood flow increases in the chorioallantoic membrane with increasing ambient (incubation) temperature. In chicken embryos the body core temperature remained constant for more than 40 minutes after the beginning of the increase in ambient temperature by activating this heat loss mechanism (Nichelmann and Tzschentke, 2003).

Endothermic reactions occurring before internal pipping were found in Muscovy duck and chicken embryos (Nichelmann *et al.,* 1998). In comparison with the heat loss mechanisms, efficiency of thermoregulatory heat production is very low. And because of the high cold tolerance of the embryos, a higher efficiency of heat production is not necessary for the survival of the bird embryos (Nicehlmann and Tzschentke, 2003; Tzschentke, 2003).

Acoustic stimulation induces clear changes in heart rate in Muscovy duck embryos from day 27 of incubation until hatching (Höchel *et al.,* 2002). The influence of ambient temperature (Ta) on temperature of allontoic fluid (Taf) is obvious. Taf is variable and depends highly on Ta (Loh *et al.,* 2004). As in birds endothermy is already developed at the end of incubation (Janke *et al.,* 2002). The dependence of Taf on Ta might be the prerequisite of a low or high embryonic temperature during the sensitive period because a low or high embryonic temperature during the sensitive period might induce a shift of the set point of the thermoregulatory system to a different level in comparison to the control group incubated at the normal temperature of 37.5°C.

In contrast to physiological thermoregulatory mechanisms, the thermoregulatory behavior (like temperature preference) in poultry hatchlings is very well developed and essential to maintain a stable body temperature.

Prenatal development of central nervous mechanisms of the thermoregulatory system

Similar to peripheral mechanisms of thermoregulation, central nervous thermoregulatory mechanisms are developed early and might show the same fundamental characteristics in prenatal conditions as experienced in the postnatal (Tzschentke *et al.,* 2004). In Musocy duck embryos thermosensitive PO/AH neurons were found on days 22 and 23, which show characteristics similar to post hatching growing (Tzschentke and Basta 2000) and adult birds (Nakashima *et al.,* 1987) as well as mammals (Schmid and Pierau, 1993). From day 28 of incubation until hatching, the proportion of cold, warm and insensitive neurons in relation to all the neurons investigated was very constant and not significantly different from that in the hatchlings (Tzschentke and Basta 2000; Maier 2003). In Muscovy duck embryos (Tzschentke *et al.,* 2004) as well as in ducklings during the first 10 days of life (Tzschentke *et al.,* 2000) and possibly in adult birds (Kanosue and Schmid, unpublished results, cited by Schmid *et al.,* 1993) thermosensitivity of PO/AH neurons can be modulated by the neuropeptide bombesin, which is know to influence thermoregulation in ectotherms and endotherms.

Altogether, during the late prenatal ontogeny in birds, especially studied in the Muscovy duck, prerequisites for central nervous control of temperature regulation are already developed. For early consolidation and maturation of body functions, sensory inputs are necessary. Environmental influences (temperature, light, acoustic signals) can stimulate this process (training effect) (Nichelmann and Tzschentke 2003).

Body functions start with uncoordinated and immediate non-adaptive reactions during early ontogeny

In the course of embryonic development, stimulation of body functions due to changes in the environmental conditions induces as a rule first uncoordinated and immediate (proximate) non adaptive-reactions. It seems that during the early development of body functions it is not important for the organism that an adaptable reaction occur but rather the fact that the reaction occurs anyway is important for the adaptability during later the life. At the end of the embryonic or during the early postnatal period a qualitative change occurs in the reaction pattern of body function after environmental stimulation. The

uncoordinated and/or immediate non-adaptive "training" reactions change into coordinated and/or adaptive reactions (Tzschentke *et al.,* 2004).

Prenatal activation pattern of central nervous mechanisms of the thermoregulatory system

Neuronal mechanisms first react on different endogenous or exogenous (sensoric) factors in an uncoordinated and immediate non-adaptive manner.

Bombesin application increases the warm sensitivity of the PO/AH neurons, followed by an increase in the activity of heat loss mechanisms, which induces a fall in body temperature in adult mammals (Brown *et al.,* 1977; Jansky *et al.,* 1987) and also in adult ducks (Schmid *et al.,* 1993) and stimulates a situation in the brain similar to hyperthermic conditions. In brain slices of 5 and 10 days old Muscovy ducklings bombesin application induced a change in the TC of the majority of the PO/AH neurons. In contrast to the mammals, in juvenile ducklings the TC increased and also decreased in an equivalent manner with respect to the temperature insensitive neurons and only a few neurons were transformed into another class of sensitivity. One explanation of these results is, that the non-specific reaction of PO/AH neurons on bombesin is typical for early ontogeny and might have changed in the course of later development. Results from *in vivo* application of bombesin on food intake and water consumption in adult ducks (De Caro *et al.,* 1980) and chicken (Denbow, 1994) support the hypothesis (Tzschentke *et al.,* 2000) that adult birds may have a mammalian like change in neuronal hypothalamic thermosensitivity after bombesin application. *In vivo* application of bombesin induced in adult ducks and chickens a decrease in food intake and mostly an increase in the water consumption, as known under hyperthermic conditions. Qualitative change in the effect of neurotransmitters on neuronal activity during early ontogeny was also found in mammals. GABA is a primary inhibitory neurotransmitter in all adult mammalian central nervous system. Electrophysiological investigations in cell cultures of embryonic and 1 to 7 days old rats have shown that in embryos, GABA application has an inhibitory as well as excitatory influence on neuronal hypothalamic activity. During advanced postnatal ontogeny the inhibitory influence becomes predominant (Chen *et al.,* 1996).

1.2 Neurotransmission and role of Gamma-amino butyric acid (GABA)

Most cells communicate via chemical synapses. Neurotransmitters are used to communicate the signal from one cell to the next. Neurotransmitters can be classified into three major groups:

1. Amino acids (primarily glutamic acid, GABA, aspartic acid & glycine).
2. Peptides (vasopressin, somatostatin, neurotensin etc).
3. Monoamines (norepinephrine, dopamine & serotonin) and acetylcholine.

Most neurotransmitters are specific for the kind of information that they are used to convey. In addition, the same neurotransmitter may elicit a variety of different responses based on the type of tissue being targeted and which other neurotransmitters, if any, are co-released. The integral role of neurotransmitters on the normal functioning of the brain makes it clear to see how an imbalance in any one of these chemicals could very possibly have serious clinical implications for an individual. Whether due to genetics, drug use, the aging process, or other various causes, biological dysfunction during synaptic transmission often leads to such imbalances and is ultimately source of conditions such as schizophrenia, Parkinson's disease, and Alzheimer's disease.

Gamma-amino butyric acid (GABA)

GABA is the chief inhibitory neurotransmitter found in the central nervous system of widely divergent species and plays a key role in modulating neuronal activity. Though reported over 50 years ago, its significance as a neurotransmitter was not fully realized until 20 years since free amino acid was positively identified in mammalian brain. Evidence from representative species in most vertebrate classes suggests that major elements of the GABAergic system have been conserved. For example, the avian brain also possesses receptors from the $GABA_A$ and $GABA_B$ families (Glencorse *et al.,* 1991). There are nonetheless significant differences in receptor structure between birds and mammals.

The hypothalamus plays an important role in regulation of a number of autonomic functions, including body temperature, food intake, cardiorespiratory activity, nociception/analgesia, circadian rhythms and the endocrine system (Meister 1993).

Between 20-50% of all central synapses use GABA as their transmitter. The formation of GABA occurs by the decarboxylation of glutamate catalyzed by glutamate decarboxylase (GAD). And the enzyme responsible for the formation of GABA from the amino acid glutamate is glutamic acid decarboxylase. Its localization and synthetic

pathway were defined and there were attempts to define its synaptic release and inactivation processes. Information on pathway specific release was difficult to define biochemically, but GABAergic projections were identified and confirmed using electrophysiological techniques.

Considerable attention was given to defining the nature of the receptor through which GABA acts and this culminated with the emergence of the structure of the ionotropic receptor (Olsen and Tobin 1990). GABA acts at inhibitory synapses in the brain. This action occurs by binding to specific receptors in the plasma membrane of both pre and postsynaptic neurons. This binding causes the opening of ion channels to allow either the flow of negatively charged chloride ions into the cell or positively charged potassium ions out of the cell. This will typically result in a negative charge in the transmembrane potential, usually causing hyperpolarization. Substances such as general anaesthetics and, later, neurosteroids were shown to potentiate the effect of GABA. But the action of the most important of these modulators, the benzodiazepines, was first described by Haefely *et al.* (1975). These important therapeutic agents act allosterically to increase the opening frequency of the GABA channel and in so doing provide a mechanism for inducing anxiolytic and sedative effects.

1.2.1 Classes of GABA receptors

The concept that GABA is a neurotransmitter in the mammalian cental nervous system is supported by both electrophysiological and biochemical data. Whereas the electrophysiological studies are essential for demonstrating a specific functional response to GABA, the biochemical approach is useful for characterizing the molecular properties of this site. $GABA_A$ and $GABA_B$ receptors are most readily distinguished by the actions of bicuculline and baclofen, the former being a $GABA_A$ receptor-specific antagonist while the latter is a $GABA_B$ receptor-specific agonist (Bowery *et al.,* 1980). Other receptor-selective agents exist, one of the most widely used of which is muscimol, a specific agonist at $GABA_A$ receptors (Krogsgaard-Larsen *et al.,* 1979).

Three general classes of GABA receptors are known. These include $GABA_A$ and $GABA_C$ ionotropic receptors, which are ion channels themselves, and $GABA_B$ metabotropic receptors, which are G protein-coupled receptors that open ion channels via intermediaries (G proteins).

The $GABA_A$ and $GABA_C$ receptors are Cl^- channels that mediate fast synaptic inhibition. Both the $GABA_A$ and $GABA_C$ receptors are members of a superfamily of

transmitter-gated ion channels that includes the nicotinic acetylcholine, strychnine-sensitive glycine and $5HT_3$ receptors. These transmitter-gated ion channels are believed to be structurally very similar, composed of five subunits that arrange together to form an ion channel. But $GABA_A$ and $GABA_C$ receptors are biochemically, pharmacologically and physiologically different (Bormann and Feigenspan 1995 and Johnston 1996).

1.2.1.1 Ionotropic receptors: GABA $_A$ receptor

These also referred to as ligand-gated channels, act quickly to depolarize the neuron and pass on the action potential (or hyperpolarize the neuron and inhibit additional action potentials). In brain, the $GABA_A$ receptor is the most broadly distributed subtypes and is responsible for many diverse and important actions in the central nervous system (Enna and Snyder 1975; Palacios and Kuhar 1980 and Olsen and Homanics 2000). Binding of the mammalian $GABA_A$ receptor agonist muscimol, occurs in most of the same regions in the quail brain as in the rat brain (Canonaco *et al.,* 1991). GABA binding to the $GABA_A$ receptor plays important roles in audition and learning and memory in birds (Fluck *et al.,* 1997). $GABA_A$ receptors are selectively blocked by the alkaloid bicuculline and are modulated by benzodiazepines, steroids and barbiturates (Bormann and Feigenspan 1995 and Johnston 1996). GABA and other directly acting $GABA_A$ receptor agonists (GABA-mimetics) bind specifically to a recognition site located at the interface between an a and a b subunit (Amin and Weiss 1993; Ebert *et al.,* 1994 and Amin, Brooks-Kayal and Weiss 1997) whereas the classical benzodiazepines, such as diazepam, flunitrazepam, as well as the novel benzodiazepine ligands with a non-benzodiazepine structure as for example zaleplon, zolpidem, zopiclone and indiplon, bind to an allosteric site located at the interface between an a and a g subunit (Amin and Weiss 1993; Amin, Brooks-Kayal and Weiss 1997 and Duncalfe 1996).

Hypothalamic neurons express functional $GABA_A$ receptor subtypes that incorporate $\alpha1$ and/or $\alpha2$ sub units, $\beta2$ and/or $\beta3$ subunits, and the $\gamma2$ subunit. Receptors expressing $\alpha3$ to $\alpha6$, $\beta1$, $\gamma1$, and δ, if present, represent a minor component of functional hypothalamic $GABA_A$ receptors (Huang and Dillon 2002). $GABA_A$ receptors consist of pseudosymmetrical, pentameric array of transmembrane subunits that form a receptor/Cl^- ion channel complex. GABA changes from an excitatory to inhibitory neurotransmitter within 2 weeks of birth, due to the reversal of the Cl^- gradient (Obrietan and van den Pol 1995; Rivera *et al.*, 1999). $GABA_A$ receptor function is allosterically modulated by a

variety of endogenous factors such as phosphorylation, pH, neurosteroids and Zn^{2+} (Moss and Smart 1996; Hevers and Lüddens 1998; Huang and Dillon 1999).

These receptors are made up of five individual protein subunits embedded in the cell membrane. These subunits have been labeled with Greek letters, such as alpha, beta, gamma and delta. It appears as though GABA requires both alpha and beta components in order to bind. $GABA_A$ receptors are typically made up of two alpha and two beta subunits among the five subunits, though the particular subunit composition often varies widely among brain regions and species.

The concept is that GABA released on to the primary afferent fibers produces a depolarization (rather than a hyperpolarization) of the terminals to decrease the evoked release of transmitter from the primary fibres due to shunting of the current in the nerve terminal. The depolarizing action would still be mediated via an increase in Cl^- conductance if the reversal potential for Cl^- in primary afferent neurons is more positive than the resting membrane potential. An increase in Cl^- flow would depolarize the neuronal membrane with a decrease in transmitter release from the primary afferent terminal.

$GABA_A$ receptors as measured by muscimol binding are found through out the hypothalamus (Xia and Haddad 1992). In situ hybridization studies have demonstrated that mRNA for α2, β3, γ2 and ε sub units are highly expressed, where as mRNA for α1, α3, α5, β1 and γ1 subunits are moderately expressed (Wisden *et al.,* 1992). Immunohistochemical studies suggest the existence of α1, α2, β2, β3, and γ2 subunits in the hypothalamic magnocellular neurons (Fenelon and Herbison 1995) and α1, α2, α5 β2/β3, and γ2 in another hypothalamic regions (Pirker *et al.,* 2000).

Ligand binding to the $GABA_A$ receptor

GABA binding (to the "GABA site") activates the $GABA_A$ receptor, allowing chloride ions to flow through the central pore and hyperpolarize the neuron, decreasing the probability that it will propagate an action potential. In this activity, the $GABA_A$ receptor does not differ from any other ligand-gated ion channels. However, among neurotransmitter receptors, $GABA_A$ receptors are unique in the number of ligands that allosterically modulate receptor function (Olsen *et al.,* 2004).

$GABA_A$ receptors can exist in at least three different conformations: open, closed, and desensitized (Sigel, 2002). Up to 14 different ligand binding sites have been proposed to account for the modulation of GABA (Tsang and Hue, 2004). Binding to the receptor can alter the conformation in such a way as to enhance or diminish the chloride flux in

response to GABA binding. Some anaesthtics (etomidate, pentobarbitone), both enhance chloride flow in response to GABA binding as well as activating it directly in the absence of GABA. Other ligands, cage convulsants of the picrotoxin type, bind within the central pore, occluding the channel and preventing chloride flow no matter what (other) ligand subsequently binds. Some of these compounds have seen commercial use as pesticides.
Classical benzodiazepines do not directly open the ion channel; rather they allosterically modify the $GABA_A$ receptor upon binding, potentiating the effect of GABA binding when there is a submaximal concentration of GABA present and thereby increasing hyperpolarizing responses and neuronal inhibition. Benzodiazepines produce systemic effects that include sedation, amnesia, muscle relaxation, and anxiolysis (Krogsgaard-Larsen *et al.,* 2002). Although the site is called the benzodiazepine site, drugs of other types can also bind and allosterically modify the receptor at that site. These include drugs with β-carboline, imidazopyridine, and triazolopyridazine structures (Sigel, 2002).

1.2.1.2 Metabotropic receptors: GABA $_B$ receptor

Bowery *et al.* (1980) were the first to discover that GABA reduces norepinephrine release by activating a bicuculline- and isoguvacine-insensitive receptor, which they named the $GABA_B$ receptor. These are also referred to as G-protein linked receptors and they do not work as simply as ligand-gated ion channels do. Like ionotropic receptors, metabotropic receptors also have an extracellular neurotransmitter recognition site, yet these receptors do not form a membrane-spanning pore that can allow the direct passage of ions. Instead, when a neurotransmitter associates with the extracellular recognition site, an intermediate molecule within the postsynaptic cell, called a G-protein, is activated and, either directly or through a series of enzymatic reactions, opens or closes ion channels located at other places on the cell membrane. Because the action of metabotropic receptors is not as direct, their action is slower. Depolarization takes longer, typically lasting up to hundreds of milliseconds, and in some cases, going on for several minutes, hours, or even days.

$GABA_B$ receptors enable GABA to modulate neuronal function in a manner not possible through $GABA_A$ receptors alone. These receptors are present at both pre- and postsynaptic sites and can exert both inhibitory and disinhibitory effects. In particular, $GABA_B$ receptors are important in regulating n-methyl D aspartate (NMDA) receptor-mediated responses, including the induction of LTP. They also can regulate the filtering properties of neural networks, allowing peak transmission in the frequency range of theta rhythm. Generally, $GABA_B$ receptors are G protein-coupled to a variety of intracellular effector systems, and thereby have the potential to produce long-term changes in the state

of neuronal activity, through actions such as protein phosphorylation. Although the majority of the effects of $GABA_B$ receptors have been reported *in vitro*, recent studies have also demonstrated that $GABA_B$ receptors exert electrophysiological actions *in vivo*. For example, $GABA_B$ receptor antagonists reduce the late IPSP *in vivo* and consequently can decrease inhibition of spontaneous neuronal firing following a stimulus (Lingenhohl and Olpe, 1993). In addition, blockade of $GABA_B$ receptors can increase spontaneous activity of central neurons, suggesting the presence of $GABA_B$ receptor-mediated tonic inhibition (Andre *et al.,* 1992; Lingenhohl and Olpe, 1993). Despite these electrophysiological effects, antagonism of $GABA_B$ receptors has generally been reported to produce few behavioral actions. This lack of overt behavioral effects most likely reflects the modulatory nature of the receptor action. Nevertheless, two separate behavioral studies have recently reported an enhancement of cognitive performance in several different animal species following blockade of $GABA_B$ receptors (Mondadori *et al.,* 1992).

Metabotropic receptors are used in the recognition of all neuropeptides and several small-molecule neurotransmitters. Dopamine (DA), for example, is a small-molecule neurotransmitter recognized by a G-protein coupled receptor. It is a member of the seven transmembrane helix-containing, guanine nucleotide-binding receptor G-protein-coupled receptors. $GABA_B$ receptors ($GABA_BR$) are metabotropic transmembrane receptors that are linked via G-proteins to potassium channels (Chen *et al.,* 2005). These receptors are found in the central and peripheral autonomic nervous system (Martin and Dunn, 2002). They can stimulate the opening of K^+ channels which brings the neuron closer to the equilibrium potential of K^+, hyperpolarizing the neuron. This prevents sodium channels from opening, action potentials from firing and so stops neurotransmitter release. Thus $GABA_B$ receptors are considered inhibitory receptors. $GABA_B$ receptors can also reduce the activity of adenylyl cyclase and decrease the cell's conductance to Ca^{2+} (Siegel *et al.,* 1999).

1.2.1.2.1 Coupling to G Proteins

Evidence for a coupling of $GABA_B$ receptors to G proteins came from the sensitivity of agonist affinity to GTP analogs. Studies using *N*-ethylmaleimide (NEM), islet activated protein (IAP), pertussis toxin, or antisense knock-down provided evidence that $GABA_B$ receptors predominantly couple to Giα- and Goα-type G proteins (Greif *et al.,* 2000). It is now well established that presynaptic $GABA_B$ receptors repress Ca^{2+} influx by inhibiting Ca^{2+} channels in a membrane delimited manner via the Gβγ subunits. Postsynaptic $GABA_B$ receptors trigger the opening of K^+ channels, again through the Gβγ subunits. This results

in a hyperpolarization of the postsynaptic neuron that underlies the late phase of inhibitory postsynaptic potentials (IPSPs). Besides modulating ion channels through Gβγ $GABA_B$ receptors activate and inhibit adenylyl cyclase via the $G_i\alpha/G_o\alpha$and Gβγ subunits.

1.2.1.2.2 Coupling to Ca^{2+} Channels

Presynaptic $GABA_B$ receptors are subdivided into those that control GABA release - autoreceptors and those that inhibit all other neurotransmitter release – heteroreceptors (Bettler *et al.,* 2004).

In most preparations, $GABA_B$ receptors mediate their presynaptic effects through a voltage-dependent inhibition of high-voltage activated Ca^{2+} channels of the N type (Cav2.2) or P/Q type (Cav2.1) (Takahashi and Tsujimoto 1998). Both types of Ca^{2+} channels are expressed in presynaptic terminals and were shown to trigger neurotransmitter release (Wu and Saggau 1997). A postsynaptic inhibition of Ca^{2+} channels by $GABA_B$ receptors was also postulated. It was shown that $GABA_B$ receptors couple to different types of Ca^{2+} channels depending on the input site (Poncer *et al.,* 2000). The inhibition of Ca^{2+} inward currents is voltage dependent and varies between 10 and 42% among studies (Chieng and Bekkers 1999). Since Ca^{2+} influx and transmitter release are correlated with a third to fourth power law, $GABA_B$ agonists frequently inhibit more than 90% of neurotransmitter release with a less than 50% inhibition of Ca^{2+} channel activity. This inhibition is modulated by the action potential frequency, where strong depolarization relieves Ca^{2+} channels from their Gβγ mediated inhibition. This particular property of presynaptic Ca^{2+} channels may differentially modulate action potential, depending on their frequency (Brody and Yue 2000). $GABA_B$ receptors are also described to either inhibit or facilitate (Shen and Slaughter 1999) L-type Ca^{2+} channels. The latter effect was shown to be indirect and to depend on protein kinase C (PKC) activity. Similarly, $GABA_B$ receptors also inhibit or disinhibit T-type Ca^{2+} channels (Futatsugi and Riviello 1998).

1.2.1.2.3 Coupling to K^+ Channels

$GABA_B$ receptors induce a slow inhibitory postsynaptic current (late IPSC) through activation of inwardly rectifying K^+ channels (GIRK or Kir3) (Schuler *et al.,* 2001). Accordingly,

$GABA_B$ induced late IPSCs can be inhibited by the Kir3 channel blocker Ba^{2+} and they usually exhibit a reversal potential similar to the K^+ equilibrium potential (Bettler *et*

al., 2004). The physiological effect of Kir3- channel activation is normally a K^{2+} efflux, resulting in a hyperpolarization. The rectification properties of synaptically evoked late IPSCs differed between studies. On the one hand, the stimulus-evoked and spontaneous late IPSCs in dopaminergic neurons are inwardly rectifying and similar to those activated by baclofen. On the other hand, baclofen also induces linear or even outwardly rectifying conductances, suggesting that channels other than Kir3 can contribute to the late IPSC. These other channels may include fast inactivating, voltagegated K^+ channels and small-conductance Ca^{2+} activated K^+ channels (SK channels). Possibly, $GABA_B$ receptors enhance the activity of SK channels by inhibiting the production of cAMP after an action potential-induced Ca^{2+} influx. In addition to the well-documented coupling of $GABA_B$ receptors to postsynaptic K^+ channels, $GABA_B$ receptors also appear to activate Ba^{2+} sensitive K^+ channels at presynaptic sites. Likely these presynaptic K^+ channels are of the Kir3 type, devoid of the Kir3.2 subunit and assembled from Kir3.1 and Kir3.4 subunits (Bettler *et al.,* 2004).

1.2.1.2.4 Coupling to Adenylyl Cyclase

All of the known nine adenylyl cyclase isoforms are expressed in neuronal tissue. Giα and Goα proteins, the predominant transducers of $GABA_B$ receptors, inhibit most of them (Simonds 1999). Many studies have reported that $GABA_B$ receptors inhibit forskolin-stimulated cAMP formation, but others also observed a stimulation of cAMP production (Bowery *et al.,* 2002 and Calver *et al.,* 2002). Giα and Goα proteins inhibit adenylyl cyclase types I, III, V, and VI, while Gβγ stimulates adenylyl cyclase types II, IV, and VII. This stimulation depends on the presence of Gsα, which results from the activation of GPCRs by, e.g., norepinephrine, isoprenaline, histamine, or vasoactive intestinal polypeptide. Therefore, the stimulatory action of $GABA_B$ receptors on cAMP levels is a consequence of G protein crosstalk and depends on the expression of adenylyl cyclase isoforms together with $GABA_B$ and Gsα-coupled GPCRs. Both the inhibition and enhancement of cAMP levels by $GABA_B$ receptor activation were confirmed *in vivo* using microdialysis. Many ion channels are targets of the cAMP-dependent kinase (protein kinase A or PKA). Accordingly, a $GABA_B$ receptor-mediated modulation of K^+ channels via cAMP was reported. Significantly, the activity of $GABA_B$ receptors on adenylyl cyclase is expected to modulate neuronal function on a longer time scale. $GABA_B$ receptors were repeatedly implicated in synaptic plasticity (Vogt and Nicoll 1999). Until recently, it was unclear whether $GABA_B$ receptors can influence plasticity processes through the cAMP pathway. Recent experiments now demonstrate that G protein-mediated

signaling through $GABA_B$ receptors retards the recruitment of synaptic vesicles during sustained activity and after short-term depression (Sakaba and Neher 2003).

The $GABA_B$ binding site

All $GABA_B$ agonists and competitive antagonists bind to the extracellular domain (ECD) of the $GABA_B(1)$ subunit, as shown for other Family 3 GPCRs as well. The ECD of $GABA_B(1)$ can be expressed as a soluble protein. The truncated protein mostly retains the binding properties of wild-type receptors, indicating that it folds independently from the transmembrane domains. The X-ray structure of periplasmic-binding proteins reveals a binding pocket that is made up by two globular lobes (lobes I and II) separated by a hinge region. The two lobes close upon ligand binding, similar to a Venus flytrap when touched by an insect. Homology models of the $GABA_B(1)$ ligand-binding domain, based on the X-ray structures of the bacterial proteins, have guided mutational analysis of the GABA binding site (Galvez *et al.,* 2000). Baclofen is a GABA analogue selective for $GABA_B$ receptors used as a muscle relaxant. However, it can aggravate absence seizures, and so is not used in epilepsy. Saclofen and phaclofen are selective antagonists.

1.2.2 GABA agonists and antagonists

1.2.2.1 $GABA_A$ agonist: Muscimol

Muscimol (agarin, pantherine) is the toxic psychoactive compound present in *Amanita muscaria.* It is the decarboxylation product of ibotenic acid. It is a specific agonist of $GABA_A$ receptors. It is the main psychoactive drug present in the mushrooms Amanita muscaria, Amanita pantherina and Amanita gemmata. It is chemically 5-amino-methyl3hydroxy isoxazole and it has been known to effect body temperature and heart rate (Osaka 2004). Muscimol has been extensively used as a lead for the design of different classes of GABA analogues. To the Isoxazoles, and in particular to Muscimol, it is possible to attribute most of the effects by the fungus in tote. Almost all the somatic symptoms (for which we have seen the possible contribution of Muscarine) coincide, as do several psychological symptoms. Regarding these last symptoms: It's nevertheless noticeable that the lack of "structured" hallucinations occurs. Such are usually reported after usage of Amanita muscaria. This fact suggests that a collateral action exists with other compounds (some of these already considered) contained in the mushroom. They could act (in respect to the Isoxazoles) by strengthening, modifying or acting directly on

the structure responsible for the psychotropic effects, or, perhaps, by modulating some of their pharmacological variables tied to the resorption or distribution.

1.2.2.2 $GABA_A$ antagonist: Bicuculline

It is a competitive antagonist of GABA receptors. Since it blocks the inhibitory action of GABA receptors, the action of bicuculline mimics epislepsy. This property is utilised in laboratories across the world in the *in vitro* study of epilepsy, generally in cortical neurons in prepared brain slices from rodents.

The action of bicuculline is primarily on the ionotropic $GABA_A$ receptor, which is a ligand-gated ion channel concerned chiefly with the passing of chloride ions across the cell membrane, thus promoting an inhibitory influence on the target neuron. Studies of the inhibition of GABA-elicited responses have shown that bicuculline (Akaike *et al.,* 1987b) shift the concentration–response curve to higher GABA concentrations but do not depress the maximal response. This receptor is the major target for bezodiazepines and related anxiolytic drugs. Sensitivity to bicuculline is defined by International Union of Basic and Clinical Pharmacology (IUPHAR) as a major criterion in the definition of $GABA_A$ receptors. However in recent years a new class of ionotropic GABA receptor, defined variously as $GABA_A$ or $GABA_C$ has been characterized, which is insensitive to both benzodiazepines and bicuculline. Bicuculline and gabazine are viewed as classic competitive inhibitors of GABA binding to the $GABA_A$ receptor, but there are indications that they can induce conformational changes in the $GABA_A$ receptor. It has been known for a number of years that bicuculline blocks currents elicited by pentobarbital (Nicoll and Wojtowicz, 1980) or alphaxalone. Bicuculline can also block activation of $GABA_A$ receptors by *n*-octanol (Arakawa *et al.,* 1992), isoflurane, or propofol. The general efficacy of bicuculline in blocking channel activation provides some circumstantial support for the idea that it acts allosterically to inhibit channel activation.

1.2.2.3 $GABA_B$ agonist: Baclofen

Chemically, baclofen is related to gamma-aminobutyric acid (GABA), a naturally-occurring neurotransmitter in the brain. $GABA_B$ receptor binding was found to be present in high amounts throughout the brain (Bowery 1993) and ionotophoretic application of baclofen depressed neuronal activity (Curtis *et al.,* 1974). Numerous studies indicated that baclofen depressed transmitter release in the central nervous system (Pierau and P. Zimmermann 1973).

Baclofen is an oral medication that relaxes skeletal muscles, the muscles that move the skeleton (striated muscle). Baclofen, acting like GABA, blocks the activity of nerves within the part of the brain that controls the contraction and relaxation of skeletal muscle. Studies on cultured sensory neurons showed that both baclofen and GABA could inhibit calcium currents and this action was resistant to picrotoxin (Dunlap 1981; Dunlap and Fischbach 1981). These neurons also expressed $GABA_A$ receptors coupled to a chloride conductance increase. Thus, a single neuron appeared to express both $GABA_A$ and $GABA_B$ receptors that initiated entirely distinct actions. Baclofen (-chlorophenyl-GABA, Lioresal) currently remains the only available $GABA_B$ medication. Baclofen, a lipophilic derivative of GABA, was synthesized in 1962 in an attempt to enhance the blood-brain barrier penetrability of the endogenous neurotransmitter. $GABA_B$ agonists showed promising therapeutic effects in a whole range of other indications, but their side effects, including sedation, tolerance, and muscle relaxation, prevented further development.

1.2.2.4 $GABA_B$ antagonist: CGP35348

3-aminopropyl- diethoxymethylphosphinic acid (CGP 35348) is in effect, an intermediate in the synthesis of the phosphinic moiety. CGP 35348-sensitive $GABA_B$ receptors were found to exist in the rat central nervous system (Gemignani *et al.,* 1994). It is a selective brain penetrant $GABA_B$ antagonist (rat cortical membranes). It has higher affinity for postsynaptic versus presynaptic receptors. CGP 35348 is somewhat less potent than (S)-(+)-2-hydroxysaclofen, but capable of crossing the blood-brain barrier. Improved version of antagonists can be derived from (3-aminopropyl)-methylphosphinic acid by extending the chain length of the P-alkyl substituent, beyond the homologous ethylphosphinic acid, or by introducing an arylalkyl substituent. CGP 35348 enhances the depolarization by increasing synaptic glutamate release via blockade of $GABA_B$ heteroreceptors (Isaacson *et al.,*1993) which might be activated during titanic stimulation. CGP 35348 tended to enhance the depolarization, by blockade of IPSPB, and this could explain the facilitatory role of the $GABA_B$ antagonist, seen using population spike analysis of LTP (long term potentiation) (Olpe & Karlsson, 1990). CGP 35348 appears to be 10–30 times more potent than the $GABA_B$ receptor blocker phaclofen. Ionophoretic and behavioural experiments showed that $GABA_B$ receptors in the brain were blocked after i.p.(intraperitoneal) administration of CGP 35348. This compound may be of considerable value in elucidating the roles of brain $GABA_B$ receptors.

As GABA effects are known to be produced through two types of receptors *viz.,* $GABA_A$ and $GABA_B$ receptors, the present study opted for agonist and antagonists against

both types of receptors. GABA binding to the classical $GABA_A$ receptor directly opens Cl^- - selective; this effect can be blocked by bicuculline. Activation of $GABA_B$ receptors by GABA or baclofen is mediated by a G protein and causes a decrease in Ca^{2+} conductance or an activation of potential or Ca^{2+} -dependent K^+ conductance, effects that can be blocked by CGP35348. Thus, in order to better understand the relationships between $GABA_A$ and $GABA_B$ receptors, and to gain insight into the function of GABAergic action in the hypothalamic neurons, electrophysiological studies were made.

1.2.3 Role of GABA in thermoregulation

In mammals, hypothalamic thermosensitivity shows a high plasticity during the developmental process and such a kind of plasticity could also be observed in birds. Central transformation of afferent information into thermoregulatory efferent signals involves different transmitter substances and modulators affecting the neuronal control (Pierau *et al.,* 1998).

Thermoeffector activity and discharge rates of warm and cold-responsive neurons in the PO/AH area of birds can also be affected by the application of the various neuroactive compounds such as amines, prostaglandins, and other substances, some of which are putative avian neurotransmitters. Compounds such as norepinephrine (NE) and epinephrine (e) have been found in significant concentratons in the avian brain. Hissa (1983) provided a summary of thermoregulatory effects of central application of several of these neurotransmitters concluding that injection of such substances as NE, 5-hydroxytryptamine (5-HT), dopamine (DA), and acetylcholine (ACh) into the PO/AH area of the rock pigeon brain overall causes hypothermia at ambient temperature (Ta) below thermoneutrality. More specifically injections of different neurotransmitters into the PO/AH of this bird produced an inhibition of shivering, a decrease in heat production, and peripheral vasodilatation leading to a reduction in body core temperature (Tb) in cold exposed individuals. The microiontophoretic application of Ach, NE, and 5-HT singly or in combination to PO/AH area of conscious Pekin ducks lead to a variety of effects on the activity of warm- and cold-responsive units, which represented 17 and 20 % respectively of 355 neurons evaluated. Ach more consistently stimulated cold units where as NE inhibited and 5-HT stimulated the majority of warm units (Sato and Simon 1988).

An important neurotransmitter GABA is supposed to take part in the neuronal control of body temperature (Bligh, 1981). GABAergic terminals and receptors are present in the PO/AH (Decavel and Van den Pol, 1990). Local microinjection studies have shown

that GABA, its agonist, and its antagonist in this area may modulate body temperature (Yakimova and Ovtcharov, 1989; Queva *et al.,* 2003; Frosini *et al.,* 2004). As such GABA was specifically examined because it is a predominant inhibitory neurotransmitter in the hypothalamus (Decavel and Pol 1990). Moreover, it is more abundant, especially in the PO/AH, than in other brain regions (Blatteis 1981). Concerning the relationship between GABA and thermoregulation, numerous reports have presented interesting data. For example, GABA in the PO/AH is reportedly involved in both heat loss and heat production responses (Bligh 1981). It is also reported that GABA affects temperature-sensitive neurons in the PO/AH in experiments using GABA agonist or antagonist in brain tissue slices (Yakimova *et al*., 1996) and anesthetized rats (Jha *et al.,* 2001). In addition microdialysis technique reported that perfusion of muscimol, $GABA_A$ agonist, into the PO/AH increased Tb in freely moving rats (Osborne *et al.,* 1994). That study concluded that hyperthermia is independent of fever or hyperactivity. Furthermore, Osaka (2004) also reported, under anesthetized conditions, that GABA-induced hyperthermia in the PO/ AH resulted from activation of heat production response (increase in O_2 consumption) but not heat loss response (no change in tail skin temperature). These results indicated that GABA in the PO/AH is involved mainly in thermogenesis and thermoregulation, especially in disinhibition of heat production.

GABA has been demonstrated in relatively high concentrations in various hypothalamic nuclei, being highest in preoptic area and anterior hypothalamic area, and appears to be mainly associated with intrinsic hypothalamic neurons (Ottersen and Storm-Mathisen, 1984). It has been suggested that short axonal GABAergic neurons may form local networks modulating afferent temperature signals with in the hypothalamus (Blatteis, 1981).

The mechanism by which GABA exerts its influence on thermoregulatory processes is unknown particularly in avian species. A number of endogenous substances such as bombesin, prostaglandin E2 (PGE2) and thyrotropin-releasing hormone (TRH), which change body temperature by central and systemic application, change either the tonic activity or the temperature coefficient (TC) or both of warm-sensitive and sometimes temperature-insensitive hypothalamic neurons (Pierau, Schenda, Konrad and Sann, 1994). Thus it appears reasonable to assume that the hypothermia induced by GABA might also be due to modulation of the hypothalamic temperature-sensitive network. However, no studies have examined the detailed influence of GABA on thermoregulation in birds.

1.3 Aim of the present study

In birds and mammals neuronal hypothalamic plasticity plays a pivotal role in thermoregulation during the developmental process. The neurons in the PO/AH are presumed to build a neuronal network. In bird species electrophysiological *in vivo* and *in vitro* studies reveal that in the PO/AH thermoresponsive neurons do exist in proportions closely similar to mammals. Most of these studies were confined to the adults in both birds and mammals and only one juvenile study was done in Muscovy duck, where it showed entirely a different pattern of development when compared to the adults. Hence another precocial species was selected to investigate if the same typical pattern of development also exists in chicken as was observed in Muscovy duck species.

Thus a lacuna exists in studies related to the neuronal hypothalamic thermosensitivity in precocial birds during the early postnatal period where majority of the changes are supposed to take place in the neuronal network. And scarce studies have been made on GABAergic substances which modulate hypothalamic plasticity in chicken.

Taking the cue, the present study makes a systematic examination of chicken hypothalamic slices with respect to temperature sensitivity and modulatory action of GABAergic substances on neuronal activity and temperature sensitivity as well. The characterization of neuronal hypothalamic plasticity aims to investigate the influence of age on thermosensitivity. To this effect a vital aspect of the design of the experiment elucidates the effect of GABA receptor agonists and antagonists on neuronal activity and temperature sensitivity in chick brain slice preparation, enabling a comparative analysis between the avian and the mammalian model.

In the ensuing studies, the related objective is to fill in the hiatus on the effect of $GABA_A$ and $GABA_B$ receptor agonists and its interaction on firing rate and temperature sensitivity of different types of neurons in the PO/AH of chicken.

2 MATERIALS AND METHODS

2.1 Experimental animals and incubation

Experiments were carried out in 5, 10, 15, 20 and 30 days old White Leghorn chickens (*Gallus gallus f. domestica)*. Fertilised eggs, obtained from a commercial stock-breeder, were incubated for 17 days according to their natural environmental conditions *viz.,* at an incubation temperature of 37.5°C, atmospheric humidity of approximately 70% and automatic mechanical turning. After 17 days of incubation, eggs were transferred into another incubator maintained at an ambient temperature of 37.5 °C, atmospheric humidity of approximately 90% till their hatch. After hatch until the day of the experiment, the birds were kept at a room temperature of 20 to 25 °C. The infra red lamp was an additional source of temperature to attain 35 °C till 10 days post hatch. The food and water are provided *ad libitum*. The standardized food is obtained from the company Sniff Spezial diiten.GmbH.

Preparation of brain slices

On the day of the experiments the chickens were decapitated and their brains were removed. The brains were removed quickly within a time span of preferably less than two minutes to avoid neuronal cell death. Within the cause of this preparation the head feathers were removed, the soft skull cap above the brain was cut circular and lifted carefully. Using a blunt spatula the brain was bent into a caudal direction by avoiding any exertion of pressure on the optic nerve fibres (*Nn. optici*). This helped to prevent injury of the hypothalamus which lies dorsal to the *Chiasma opticum*. Optic nerve fibres as well as the *Medulla oblongata* were cut and the brain is transferred into cooled (4 - 6°C) and carbogenised (95% O_2, 5 % CO_2) artificial cerebrospinal fluid (ACSF). The aim of this cooling was to slow down metabolic processes and to achieve better stability of tissue to facilitate the following preparation.

After 2 minutes the brain was removed from the fluid, transferred into a plastic dish and fixed by tissue-glue with its caudal side facing downwards. Finally the dish was filled with ACSF until the brain was completely covered.

For the preparation of brain slices a vibratome with high frequency and low amplitude was used (mechanical workshop MPI Bad Nauheim). This helped to prevent damage of brain tissue during the course of preparation. Slices were of a recommended 400 µm thickness (Burgoon *et al*., 1997) and comprised the preoptic area of the anterior hypothalamus (PO/AH). They were divided along the third ventricle and placed in an

incubation chamber for at least 2 hours before recording. The chamber contained 250 ml of ACSF oxygenated by bubbling with 95% O_2, 5% CO_2 and kept at 34.5°C by immersion in a water bath. Thus thermal stabilisation after previous thermal instabilities could be achieved, likewise unstable cellular processes due to mechanical stimulation within the course of preparation could normalise.

Perfusion solutions

The composition of the perfusion solution artificial cerebrospinal fluid (ACSF) was equivalent to cerebrospinal fluid (Alger *et al.,* 1984). It was prepared daily from four concentrated stock solutions and contained:

Solution 1	NaCl	124.0	mM
	KCl	5.0	mM
	$NaH_2PO_2 \cdot H_2O$	1.2	mM
	$MgSO_4 \cdot 7H_2O$	1.3	mM
Solution 2	$NaHCO_3$	26.0	mM
Solution 3	$CaCl \cdot 2H_2O$	1.2	mM

To prevent impurity through bacterial growth solution four was freshly prepared each day.

Solution 4	$C_6H_{12}O_6$	10.0	mM

Substances were dissolved in distilled water (Millipore), osmolality of the ACSF was approximately 303 $\pm$ 2 mOsm/kg. All solutions were mixed and oxygenated for at least 1 hour with 95% O_2, 5% CO_2 before use, thereby achieving a stable pH of 7.4. ACSF was continuously oxygenated throughout the experiment.

2.2 Experimental set up for extracellular recordings

Recording chamber

Single slices were transferred into a recording chamber (Figure 3), especially conceived for *in vitro* investigations of thermosensitivity in neurons (Schmid and Pierau 1993). The chamber made from solid brass, consisted of an outer and an inner compartment and was entirely gold plated to prevent reactions between ACSF and the surface of the recording chamber. The dimension of the chamber was 40 x 40 mm, exactly fitting a powerful Peltier element (75W, Cambion, Cambridge, MA). Heat was removed by a continuous stream of tap water at the lower side of the element. Due to the close connection and the thin metal bottom of the chamber (2 mm), rapid and exact temperature changes could be achieved.

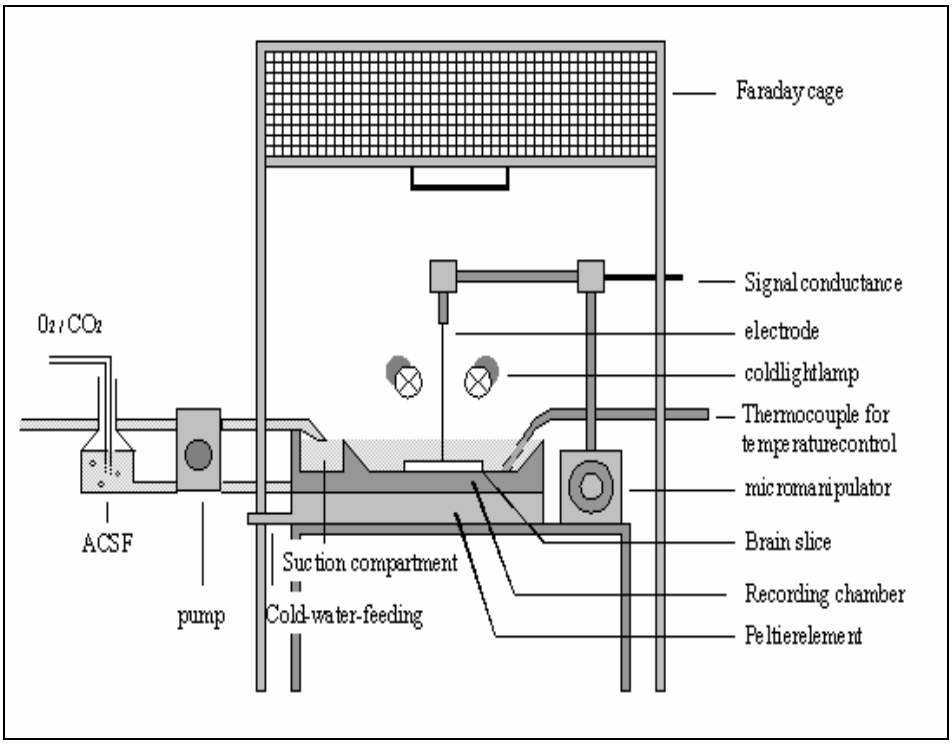

Figure 3: Experimental setup.

At the base of the chamber high-grade steel tubes were integrated in which ACSF circulated, permanently temperated with the help of the peltier device. The ACSF was led through a small opening into the recording chamber where the totally submerged slice was held in a stable position with the aid of a small platinum ring. Temperature of ACSF was continuously measured through a small thermocouple positioned directly besides the brain slice.

Continuously oxygenated ACSF flowed through polyethylene tubing (AbiMed Minipuls), after entering the chamber, through stainless steel coils in the gold-plated inner compartment. In-flowing ACSF held the same temperature as the solution already present in the inner compartment. The flow rate (2.5 ml/min) was kept constant with a plastic valve (Regu Flo, Peter von Berg, Munich, Germany). The volume of the solution filled inner chamber (0.5 – 0.7 ml) and the chosen flow rate resulted in a fluid turnover of 3-5 times/minute.

Perfusion solutions were aspirated from the outer chamber and indirectly via a steel tube from the inner chamber. This arrangement abolished possible disturbances caused by suction. Electrodes were inserted vertically by a micro-manipulator under optical guidance of a binocular (4x10/Zeiss) and two cold-light lamps.

2.3 Electrical recordings of single neurons

Single slices were transferred into the recording chamber and spontaneously active neurons were selected at a basic temperature of 40°C in the recording chamber. The basic temperature in the recording chamber was kept constant at 40°C (this bath temperature approximately corresponds to the deep body temperature of juvenile chicks, Tzschentke and Nichelmann, 1999). Single units were recorded from neurons located in the PO/AH region lateral to the third ventricle and caudal to the *Commissura anterior* and especially the area containing paraventricular nucleus (PVN) (Figure 4).

Legend

AC	Commissura anterior
AM	Nuc. anterior medialis hypothalami
BPC	Nuc. pallial commissura
CO	Chiasma opticum
DSD	Decussatio supraoptica dorsalis
DSV	Decussatio supraoptica ventralis
LFB	Fasciculus telencephalicus lateralis
LHy	Nuc. lateralis hypothalami
PVN	Nuc. paraventricularis
QF	Tractus quintofrontalis
SM	Nuc. septalis medialis
VLT	Nuc. ventrolateralis

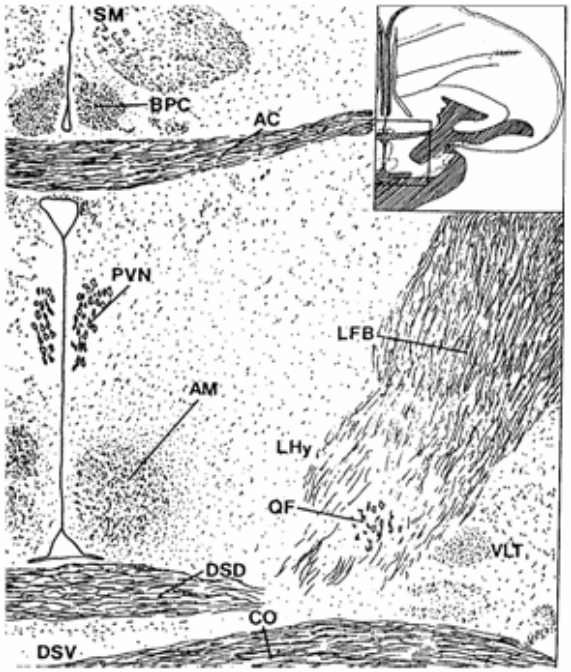

Figure 4: **Preoptic region of the anterior hypothalamus (Kuenzel and van Tienhoven 1982).**

For the extracellular recordings of the neuronal activity glass electrodes were used. The whole chamber served as a reference electrode and was grounded.

Recorded action potentials (APs) were preamplified (World Precision Instruments, DAM5A), filtered (0.1-3kHz band pass) and displayed on a storage oscilloscope. The AP amplitude exceeded the noise (25 μV) on average 5-20 fold. AP events were detected by a window discriminator (World Precision Instruments, model 120) and counted by second with a rate meter. Data were stored simultaneously on a chart recorder (Hellige Recomed 218100) and on a computer (Figure 5).

Neuronal activity and slice temperature were recorded by conventional electrophysiological equipment and stored on a personal computer using a 1401 interface (Cambridge Electronic Design (CED)) and the CED software Spike 2. Only activity of single units, which were separated using a window discriminator, were stored on the

computer. Changes in neuronal tonic activity (firing rate) were calculated with the aid of the same computer program, providing information on the mean value of firing rate for the duration of 1 minute, recorded just prior to each temperature stimulus.

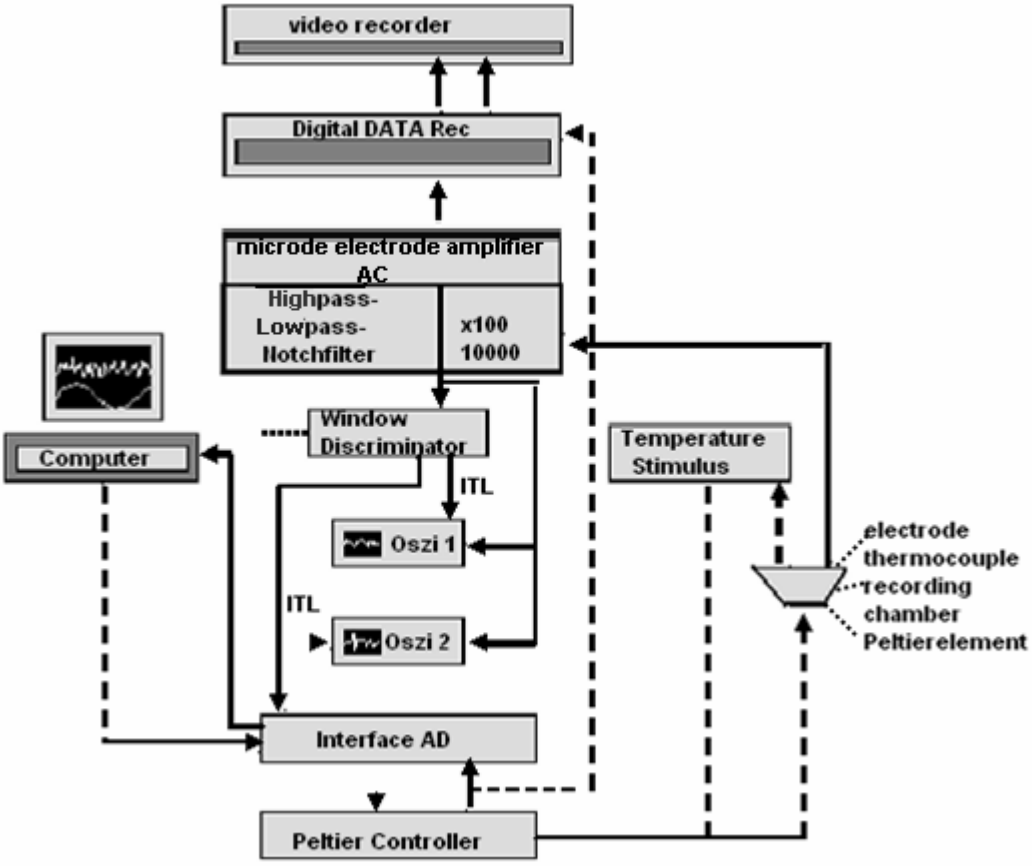

Figure 5: Signal processing.

2.4 Temperature stimulation

The temperature was changed sinusoidal by a computer-based stimulus generator (± 3°C) with a velocity of 0.02°C/s (Schmid and Pierau 1993), (Figure 6).

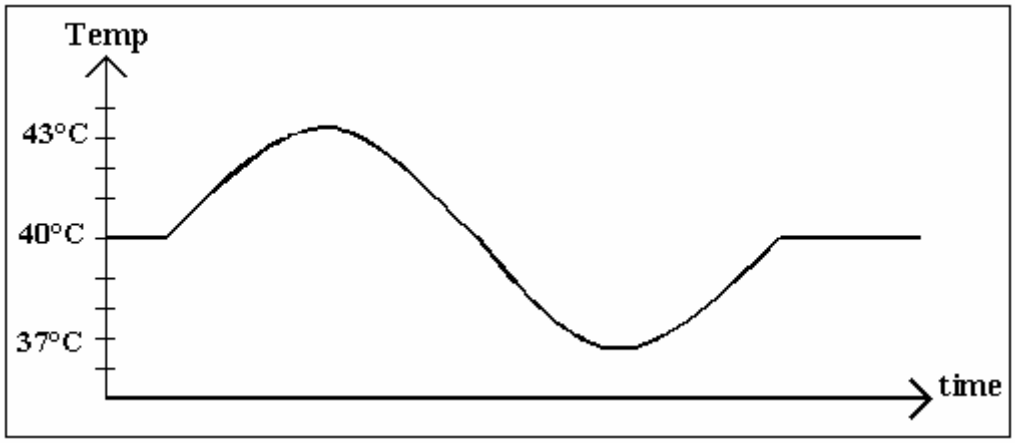

Figure 6: Sinusoidal temperature change (± 3°C).

The stimulus generator controlled the Peltier temperature control unit which changed the temperature sinusoidal with the above mentioned velocity without any overshoot of the controlled temperature.

Starting at 40°C the temperature increased to 43°C, then fell continuously back to 37°C and reached the basal temperature after approximately 13 minutes. These values are according to normal changes that can occur in homeothermic animals during motoric activation and in high or low ambient temperatures (Abrams and Hammel 1964; Meisenberg and Simmons 1984; Kruk *et al.,* 1985). The Peltier controller in combination with the stimulator allowed repetition of precisely the same temperature stimuli throughout the experiment. After recording the spontaneous activity of a neuron for 5 minutes at 40°C, the temperature sensitivity was tested with at least two temperature cycles. In between both cycles spontaneous activity was again recorded at 40°C for at least seven minutes.

2.5 Analysing procedure of neuronal thermosensitivity

The temperature response curve of each neuron was evaluated by a computer program (Spike2 - Cambridge Electronic design limited) that fitted a piecewise linear regression function as well as a rectilinear regression function over the time period of a complete temperature cycle (Vieth 1989). The piecewise analysis of the frequency/temperature relation allows the statistical estimation of the intersection of two regression lines without requiring further preconditions. The program determines the intersection of two regression lines by an iterative approach that successively fits two regression lines to the data points of a given temperature stimulus. It calculates the residual sum of squares of both regression

lines and selects the fit that causes the minimum error as the optimal fit for the data. With this statistical method the physiological "threshold" can be determined, i.e., the point above or below which a neuron becomes temperature sensitive. Thus, the range of its highest temperature sensitivity can also be determined. If the difference between the residual sum of squares of one or both regressions was >10%, the fit of both regressions was used to determine the range of the highest temperature coefficient (TC) of a neuron. Neurons were classified according to the slope of the regression line in the temperature range where their TC was highest, provided these slopes covered a temperature range >2°C.

Cells with a positive or negative TC value larger than or equal to 0.6 imp/s/°C were classified as being warm-sensitive or cold-sensitive respectively. All other cells were regarded as temperature insensitive by this definition (Nakashima *et al.,* 1987).

2.6 Protocol of synaptic blockage and application of GABAergic substances (perfusion solutions)

A typical protocol has been designed to test the effect of different perfusion solutions (with different substances) on the neuronal action (figure 7). A synaptic blockade was performed in which calcium free ACSF was used as the perfusion solution during a particular temperature stimulus as required. To test the effect of GABAergic substances on the neuronal action, the designated GABAergic substance was dissolved in the normal ACSF in premeditated concentrations during a particular temperature stimulus as required.

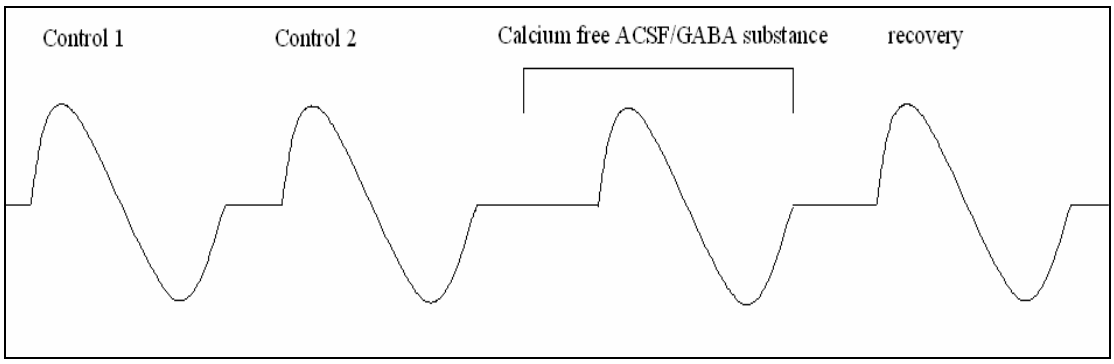

Figure 7: A typical protocol of the sinuses under different actions viz., controls and superfusion under different substances (calcium free ACSF/GABAergic substances) and a recovery sinus.

Synaptic blockade

Calcium free ACSF was used as a perfusion solution to test the inherent tendency of a cold-sensitive neuron. Initially the neuron is recorded for 5 minutes (until it stabilizes) and then the temperature stimuli are given as required. Superfusion was made with normal ACSF for two temperature stimuli to analyze the sensitivity of the neuron. Later normal ACSF was allowed for 5 minutes and another temperature stimulus was given during the application of calcium free ACSF to test the inherent tendency of the neuron. Finally a recovery sinus is given under normal ACSF after a prolonged washout (figure 7). Preferentially some of the cold-sensitive neurons were blocked by calcium free ACSF and also simultaneously with GABAergic substances.

Application of GABAergic substances

$GABA_A$ receptor agonist muscimol and it's antagonist bicuculline and $GABA_B$ receptor agonist baclofen and its antagonist CGP35348 were used in the present series of experiments to test the effect of GABAergic action in different doses (figure 7).

The $GABA_A$ receptor agonist muscimol hydrobromide (1 μM) (Sigma-Aldrich GmbH), $GABA_A$ receptor antagonist bicuculline methoidide (10 μM) (Sigma-Aldrich GmbH), $GABA_B$ receptor agonist R(+)-baclofen hydrochloride (1 μM) (Sigma-Aldrich GmbH) and $GABA_B$ receptor antagonist CGP 35348 (10 μM) (Sigma-Aldrich GmbH) were diluted in ACSF just prior to the application. The concentrations of the substances used in this investigation are selected on the basis of previous standard literature. These concentrations have showed the best effects in previously made investigations on the influence of GABAergic substances on rat PO/AH neurons (Yakimova *et al*., 1996). Before application of the test substances, the temperature sensitivity of a given neuron was determined using two sinusoidal temperature stimuli at intervals of 5 minutes. Superfusion of test substance was started not before 5 min after the last control temperature stimulus; the test substances were applied for 5 min before the next temperature stimuli were performed. Superfusion returned to ACSF after this stimulus was completed and a further temperature stimulus was given after a delay of at least 10 min. An additional temperature stimulus was applied in anticipation of complete recovery. In order to assess agonist-antagonist interactions, the appropriate antagonist in tenfold higher concentration was superfused simultaneously with the agonist. Only one neuron per slice was tested.

Investigations were carried out in two series of experiments. In the first series of investigations, the effect of the $GABA_B$ receptor agonist baclofen in 35 chick hypothalamic

neurons has been studied. In the second series of experiments the effect of synaptic blockade under calcium free ACSF on cold-sensitive neurons and the effect of GABAergic substances has been studied independently as mentioned under their sub headings respectively (125 chick PO/AH neurons were exclusively used in studies related to GABAErgic investigations and 13 cold-sensitive neurons were investigated under different conditions of synaptic blockade).

2.7 Statistics

The proportion of warm-sensitive, cold-sensitive and temperature insensitive neurons in the PO/AH was determined in relation to all neurons investigated in chicken. For instance, an increase in the proportion of cold-sensitive neurons and a decrease in warm-sensitive neurons in relation to all neurons investigated was used as a sign of elevation in total neuronal cold-sensitivity of the PO/AH. For statistical evaluation chi-square (χ^2)-test was employed to test for differences between different age groups.

In order to compare the results between different age groups independent of the classification of neurons as temperature sensitive or insensitive by the limitation value of the TC, the relative frequency of TC of all PO/AH neurons was investigated (bin width 0.1 imp/ · s/°C). Kolomogorov-Smirnoff's test and subsequently U-test were employed to test for differences between frequencies for normal distribution.

In relation to the GABAergic substances, all the data are presented as means means ± S.E.M. For statistical evaluation a paired t-test was performed.

3 RESULTS

3.1 Plasticity of chick hypothalamic neurons: influence of age

Characterization of temperature sensitive and insensitive neurons in 5, 10, 15, 20 and 30 – days old chickens has been performed. In the present series of experiments the proportion of warm-sensitive, cold-sensitive and temperature insensitive neurons in relation to all neurons investigated in each age group in the PO/AH of the chicken has been calculated and given in table 1.

Table 1: Type, number and percentage of neurons in the PO/AH in 5 to 30 days - old chickens in relation to all the neurons investigated in each group.

Age groups	Type of neurons	Number of neurons	Percentage of neurons
5 days	Warm sensitive	15	22
	Cold sensitive	24	35
	Insensitive	29	43
10 days	Warm sensitive	14	19
	Cold sensitive	23	32
	Insensitive	35	49
15 days	Warm sensitive	17	21
	Cold sensitive	34	43
	Insensitive	29	36
20 days	Warm sensitive	13	18
	Cold sensitive	38	52
	Insensitive	22	30
30 days	Warm sensitive	24	29
	Cold sensitive	28	34
	Insensitive	30	37

Development of neuronal hypothalamic thermosensitivity from day 5 to day 30 post hatching has been shown in figure 8 in relation to the investigated neurons *viz.,* cold-, warm- and insensitive neurons.

3.1.1 Comparison of neuronal thermosensitivity in relation to all age groups investigated

In all the age groups investigated a predominance of cold-sensitive neurons has been observed in relation to thermosensitive neurons but a shift towards neuronal warm sensitivity occurs at a later stage between 20 and 30 days.

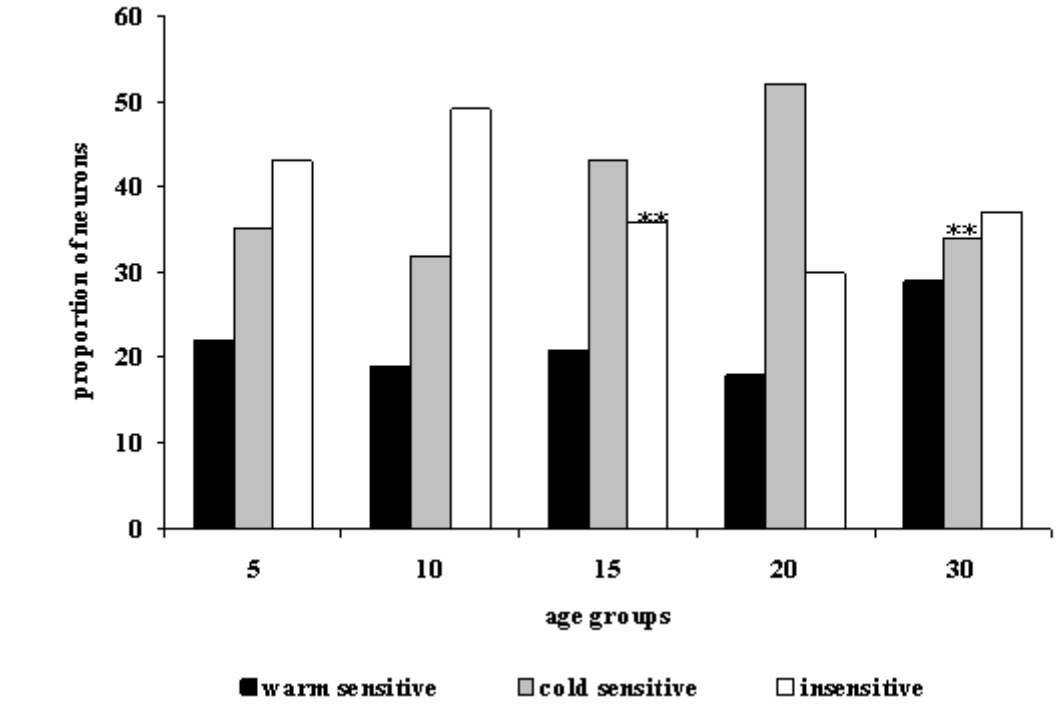

Figure 8: Influence of age on proportion of warm, cold and temperature insensitive neurons in relation to all neurons investigated in each group in the preoptic area of the anterior hypothalamus of chicken. χ^2- test at *p<0.05; **p<0.01; ***p<0.001.

An increase in the number of cold-sensitive neurons from day 5 post hatching to day 20 post hatching has been observed with an exception on day 10 post hatching, where a slight decline is exhibited (figure 8). Later there is a dramatic decline in neuronal cold sensitivity on day 30 post hatching. A comparison made in case of cold-sensitive neurons between day 20 and 30 post hatching showed a significant difference of $p<0.001$ (χ^2- test).

In the observed age groups an increase of warm-sensitive neurons has been observed on day 30 post hatching when compared to the rest of the age groups but it is not significant (χ^2- test). Temperature insensitive neurons showed a decline on day 15 and 20 post hatching when compared with day 5 and 10 post hatching. A comparison made in case of temperature insensitive neurons between day 10 and 15 post hatching exhibited a

significant difference of $p<0.01$ (χ^2- test). But there is an increase in insensitive neurons on day 30 post hatching which is also not significant (χ^2- test).

Simultaneously a comparison was made in the groups of warm, cold and insensitive neurons with 5 days in respect of the other age groups in the order as mentioned in table 2.

Table 2: Depiction of proportion of neurons in different age groups in relation to sensitivity include a comparison made with 5 days in respect of other age groups. NS represents non significant differences between the groups and p values represent significant differences between the groups (χ^2- test).

	5 and 10 days	**5 and 15 days**	**5 and 20 days**	**5 and 30 days**
Warm sensitive	NS	NS	NS	NS
Cold sensitive	NS	NS	$p<0.05$	NS
Insensitive	NS	NS	NS	NS

In relation to warm-sensitive neurons, a comparison between 5 days and 10 days; 5 days and 15 days; 5 days and 20 days and finally between 5 days and 30 days showed no significant differences (χ^2- test).

In relation to cold-sensitive neurons, a comparison between 5 days and 10 days; 5 days and 15 days; 5 days and 30 days exhibited no significant differences (χ^2- test) but a significant difference of $p<0.05$ (χ^2- test) was exhibited between 5 days and 20 days.

In relation to insensitive neurons, a comparison between 5 days and 10 days; 5 days and 15 days; 5 days and 20 days and finally between 5 days and 30 days, no significant differences (χ^2- test) were observed.

3.1.2 Distribution of different thermosensitive neurons in relation to sensitivity of neurons in individual age groups

A comparison was made in case of warm-sensitive, cold-sensitive and temperature insensitive neurons (proportion) in 5 days, 10 days, 15 days, 20 days and 30 days age groups to find the significance levels. These significance levels are described accordingly in table 3.

Table 3: Depiction of proportion of neurons in 5 days, 10 days, 15 days, 20 days and 30 days age groups in relation to sensitivity. NS represents non significant differences between the groups and p values represent significant differences between the groups (χ^2- test).

	Warm and Cold	**Warm and Insensitive**	**Cold and Insensitive**
5 days	NS	$p<0.05$	NS
10 days	NS	$p<0.001$	$p<0.05$
15 days	$p<0.01$	$p<0.05$	$p<0.05$
20 days	$p<0.001$	NS	$p<0.01$
30 days	NS	NS	NS

In 5 days old age group, during a comparison between warm and cold; between cold and insensitive neurons, no changes in significant differences (χ^2- test) were found but a significant difference of $p<0.05$ (χ^2- test) has been observed in comparison between warm and insensitive neurons.

In 10 days old age group, a comparison between warm and cold-sensitive neurons, no significant differences (χ^2- test) were found but between warm and insensitive neurons a significant difference of $p<0.001$ (χ^2 - test) has been exhibited. A significant difference of $p<0.05$ (χ^2 - test) was seen between cold and insensitive neurons in this age group.

In 15 days old age group, during the comparison between warm and cold-sensitive neurons a significant difference of $p<0.01$ (χ^2 - test) was noticed and a significant difference of $p<0.05$ was observed between warm and insensitive and as well between cold and insensitive neurons.

In 20 days old age group a significant difference of $p<0.001$ (χ^2 - test) was observed between warm and cold and $p<0.01$ (χ^2- test) in case of cold and insensitive neurons but no significant differences (χ^2 - test) were noticed between warm and insensitive neurons.

In 30 days old age group, no significant differences (χ^2 - test) were observed when a comparison was made between warm and cold; warm and insensitive and finally between cold and insensitive neurons.

3.1.3 Distribution of TC values in all the age groups investigated

A comparison between the different age groups *viz.,* 5 days, 10 days, 15 days, 20 days and 30 days in relation to the frequency of the TC values (during all temperature stimulations) of all PO/AH neurons investigated has been made as shown in the figures 9 to 13. The present comparison enables to show if there exists a normal distribution as is the case in Muscovy duck (Tzschentke and Basta 20042) where it is used as a parameter for compaarison during the process of development. The relative frequency of the TC values of all PO/AH neurons was investigated in the mentioned age groups where 'n' is the total number of individual TC values of neurons (during all temperature stimulations) investigated in each age group with a bin width of 0.1 imp/s/°C.

Differences within the relative frequency of the TC of all PO/AH neurons investigated between 5 days and 10 days; 5 days and 15 days; 5 days and 20 days and finally between 5 days and 30 days were not significant (U- test at $p<0.05$).

Similarly, differences within the relative frequency of TC of all PO/AH neurons were tested between 10 days and 20 days; 15 days and 20 days; 20 and 30 days; 5 days and 30 days; 5 days and 20 days and finally between 5 days and 15 days. During the process no significant differences have been observed for normal distribution (U – test at $p<0.05$).

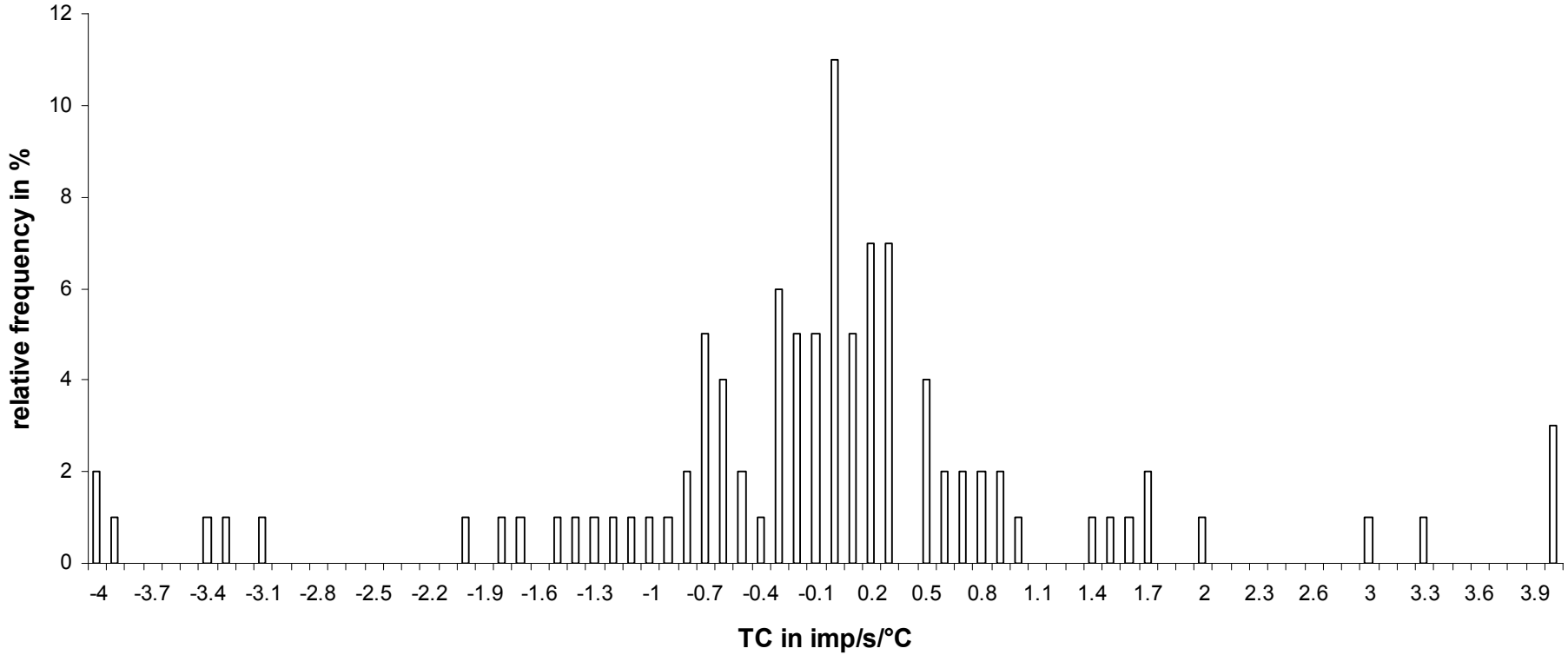

Figure 9: Distribution of TC values of PO/AH neurons in 5 days old chickens (bin width =0.1 imp/s/°C, n=130).

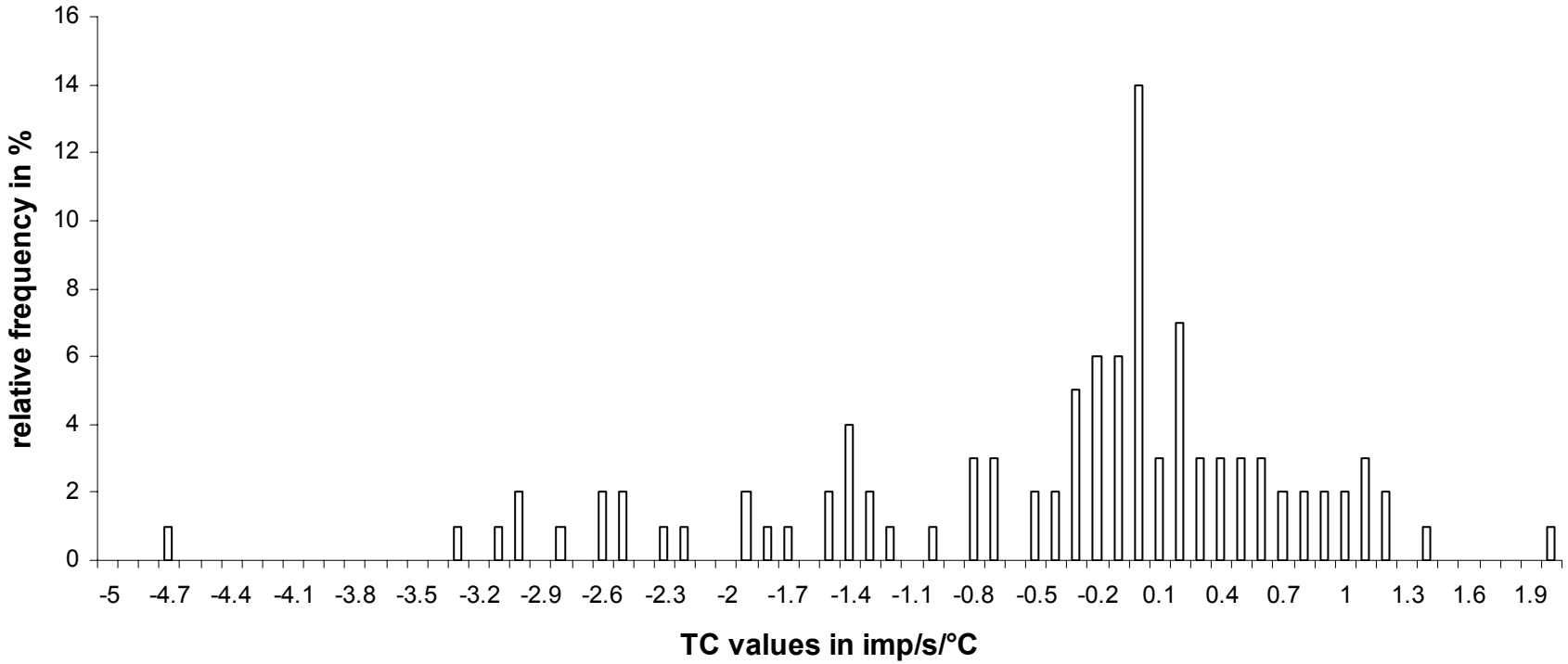

Figure 10: Distribution of TC values of PO/AH neurons in 10 days old chickens (bin width =0.1 imp/s/°C, n=116).

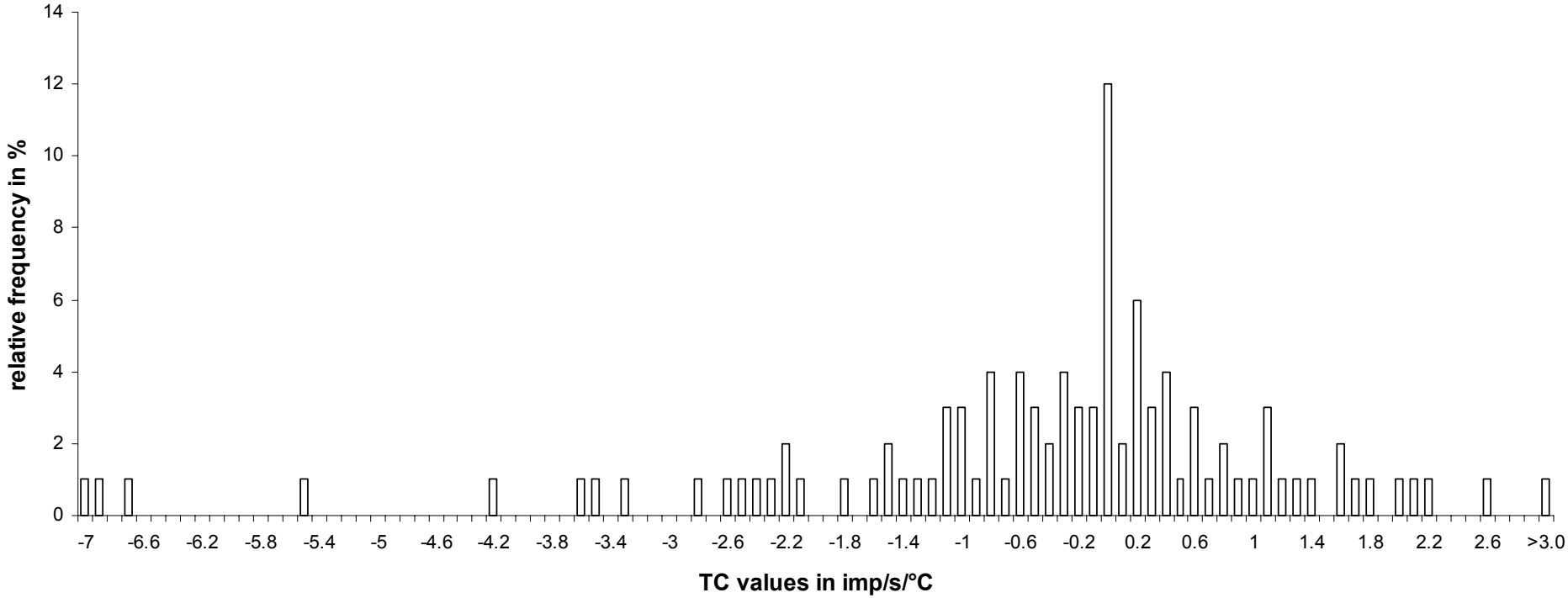

Figure 11: Distribution of TC values of PO/AH neurons in 15 days old chickens (bin width =0.1 imp/s/°C, n=158).

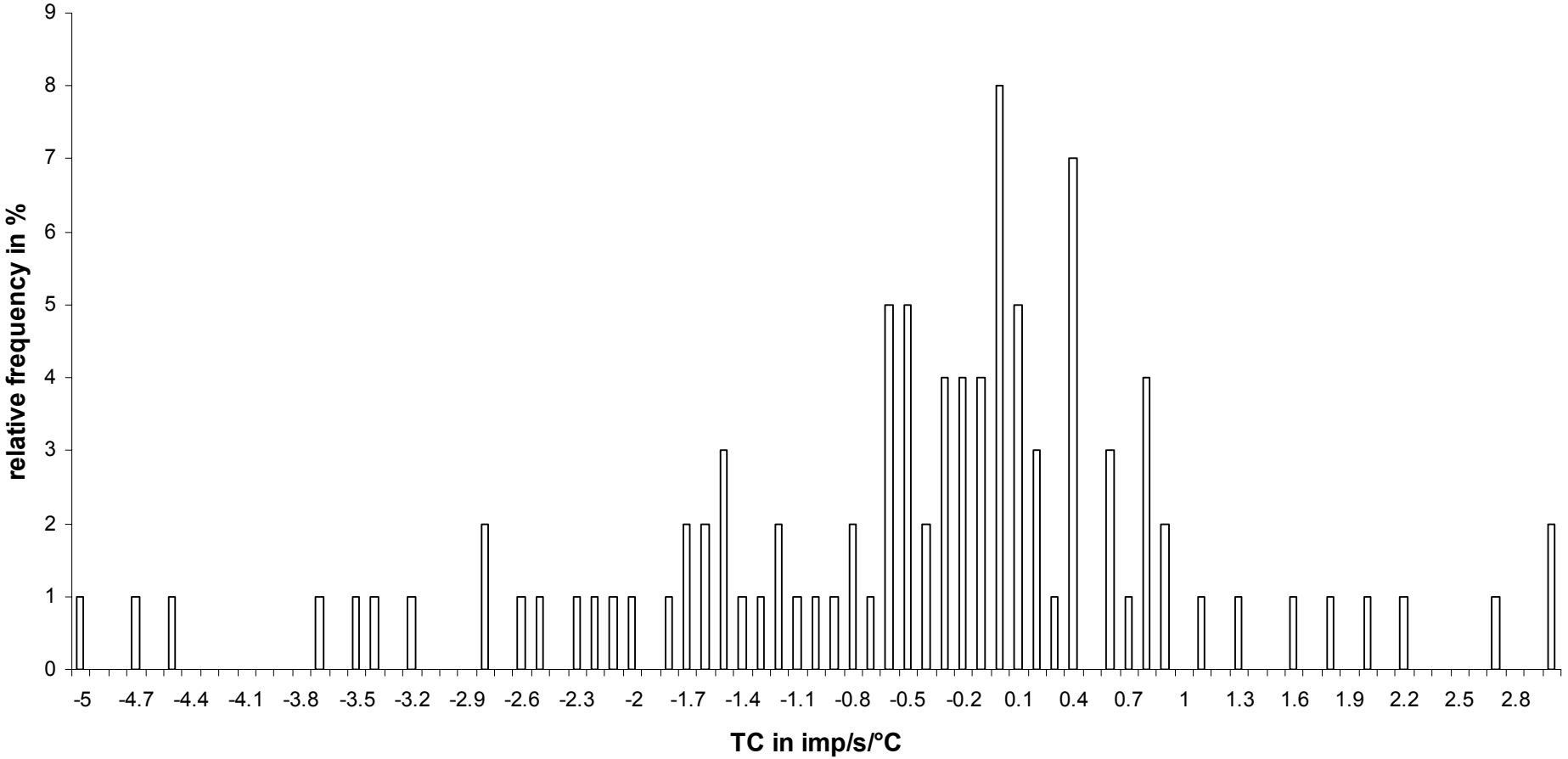

Figure 12: Distribution of TC values of PO/AH neurons in 20 days old chickens (bin width =0.1 imp/s/°C, n=137).

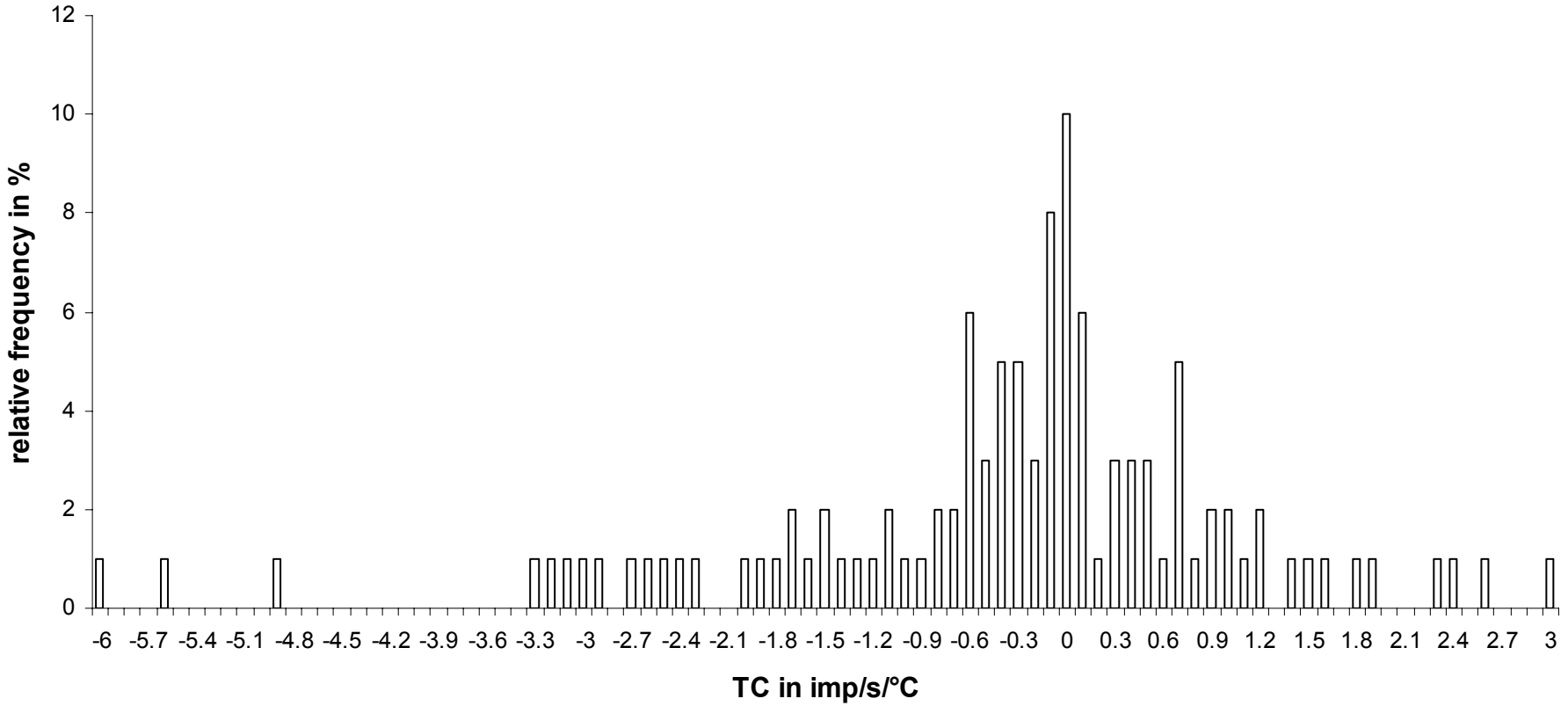

Figure 13: Distribution of TC values of PO/AH neurons in 30 days old chickens (bin width =0.1 imp/s/°C, n=145).

3.2 Characterization of inherently cold-sensitive neurons under synaptic blockade with Calcium free ACSF and under the action of GABAergic substances

Characterization of cold-sensitive neurons under synaptic blockade and also under the action of different GABAergic substances has been performed (table 4).

Table 4: Total number of cold-sensitive neurons investigated under the action of different kinds of substances include the action under synaptic blockage (calcium free ACSF) and under the action of GABAergic substances.

Action of Substances		Individual number of neurons	Total number of neurons
Influence of synaptic block			
Calcium free ACSF			13
Influence of GABAergic substances			
$GABA_A$ receptors	Muscimol	7	17
	Bicuculline	7	
	Muscimol + Bicuculline	3	
$GABA_B$ receptors	Baclofen	18	26
	CGP35348	6	
	Baclofen + CGP35348	2	

A total of 56 cold-sensitive neurons have been investigated under the action of synaptic blockade and GABAergic substances in 10 - to 20 - days old chickens in the PO/AH region. Initial studies were performed under synaptic blockage with calcium free ACSF followed by the studies under the action of different GABAergic substances v*iz.,* $GABA_A$ and $GABA_B$ receptor agonists muscimol and baclofen and their respective antagonists bicuculline and CGP35348.

To test if inherently cold-sensitive neurons in the PO/AH exist, 13 cold-sensitive neurons of 10 - to 20 - days old chickens were investigated under synaptic blockade with calcium free ACSF in the present study. Within the investigated cold-sensitive neurons, 6 increase the firing rate and two of them strongly under cold load during synaptic blockage, thus exhibiting an inherently cold sensitivity. And 7 neurons were totally blocked under synaptic blockage. All the neurons showed recovery after wash out subsequently.

A cold-sensitive neuron under synaptic blockade with calcium free ACSF which shows inherent tendency has been shown in figure 14.

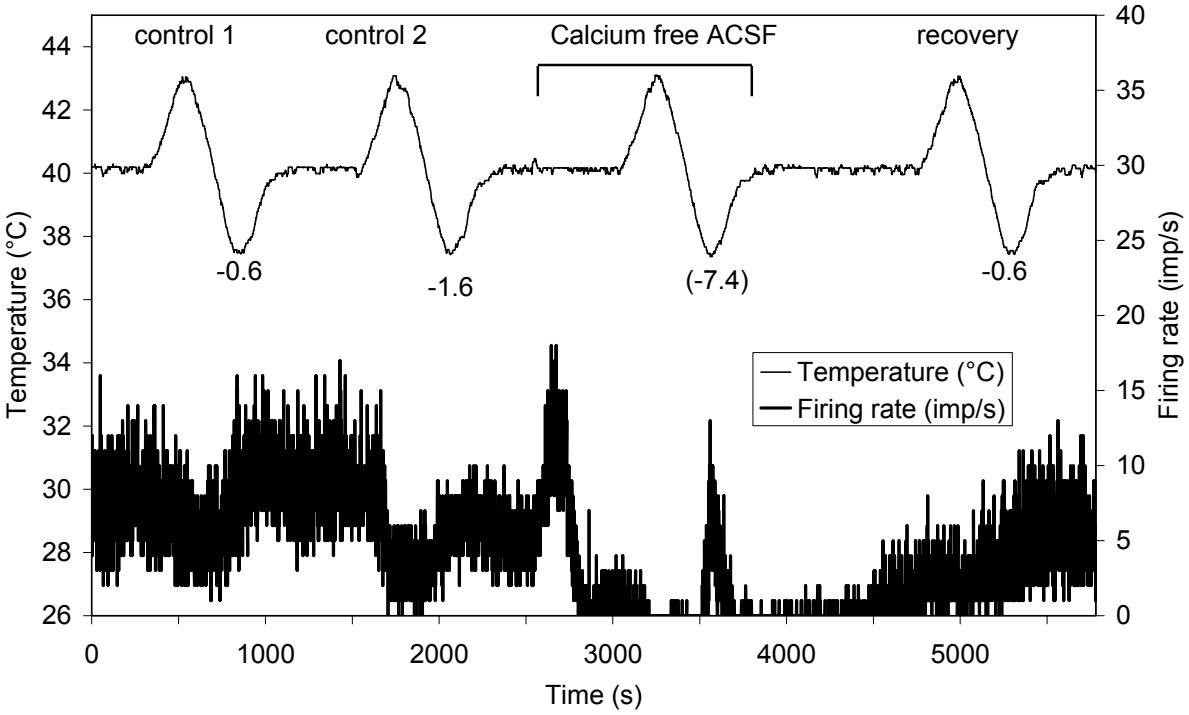

Figure 14: Example of a cold-sensitive hypothalamic neuron under synaptic blockade with calcium free ACSF which exhibits activity. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C); two control stimuli, sinus under the action of calcium free ACSF and a recovery sinus. Lower panel: Firing rate in imp/s.

In figure 14, under two control stimuli, neuron is cold-sensitive in nature. During superfusion with calcium free ACSF, the neuronal activity is inhibited. This state of inactivity of neuron persists till the cold phase of the neuron. The neuron exhibits activity in the cold phase during a short interval of time. This activity has been observed at the lowest temperature of the cold phase of the sinus. This inherent tendency of the neuron which shows activity during cold phase has been analysed for the TC value during this short interval of time as a specific case study. The calculated TC of (-7.4) over a range of 0.75 °C has been appropriately presented at its temperature stimulus. The TC has been given in brackets as it has been analysed over a range of 0.75 °C temperature change only. This has been done under special condition to analyse the inherent tendency exhibited during a short interval of temperature change instead of the normal 2 °C temperature change as described in the 'materials and methods' section. Subsequently after this brief cold phase, the neuron does not show any activity. The neuron again exhibits activity only after a thorough washout with normal ACSF. During the recovery sinus, the neuron remains cold-sensitive in nature.

A cold-sensitive hypothalamic neuron under synaptic blockade with calcium free ACSF which exhibits total inhibition during the sinus has been shown in figure 15.

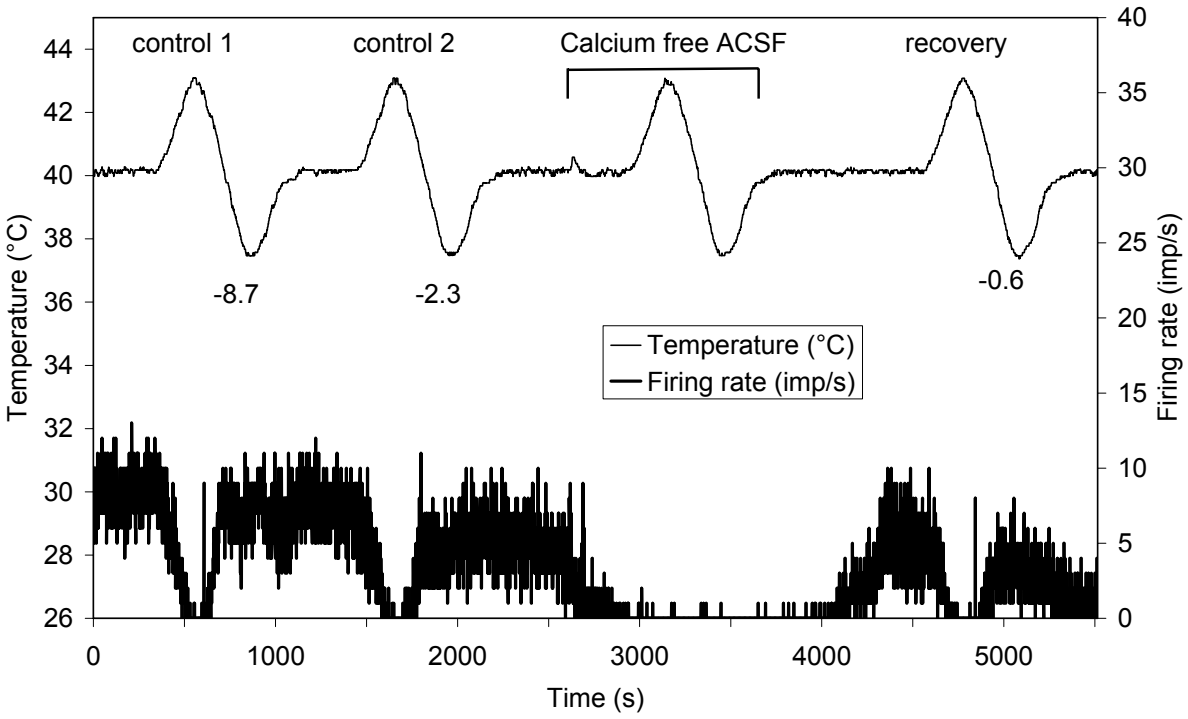

Figure 15: Example of a cold-sensitive hypothalamic neuron under synaptic blockade with calcium free ACSF which exhibits total inhibition during the sinus. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C); two control stimuli, sinus under the action of calcium free ACSF and a recovery stimulus. Lower panel: Firing rate in imp/s.

In figure 15, the neuron exhibits cold sensitivity in both the control sinuses (normal ACSF). During synaptic blockage, the neuron is totally suppressed and no firing rate has been exhibited. Subsequently TC value during synaptic blockage could not be evaluated. After washout, a recovery sinus is made which retains its cold sensitivity.

Action of different GABAergic substances *viz.,* $GABA_A$ and $GABA_B$ receptor agonists and their respective antagonists was investigated in 43 cold-sensitive neurons. Influence of $GABA_A$ agonists and its antagonists was studied in 17 neurons. Action of muscimol was studied in 7 cold-sensitive neurons and a similar number was studied under the action of bicuculline. Studies with co-application of muscimol and bicuculline were carried out in 3 cold-sensitive neurons. Spontaneous activity was reduced in all the cold-sensitive neurons during the application of muscimol (1 μM) and the TC values could not be evaluated. During the application of bicuculline (10 μM) all the neurons showed an increase in firing rate and a significant difference of $p<0.05$ (Paired t-test) was found. TC

values showed a significant difference of $p<0.05$ (Paired t-test) during bicuculline application (10 μM). Superfusion of both muscimol (1 μM) and bicuculline (10 μM) did not show significant differences (Paired t-test) either in firing rate or TC values. These results have been further described in detail under the sub heading 'Plasticity of chick hypothalamic neurons: effect of GABAergic substances' in the results part.

Influence of $GABA_B$ receptor agonists and its antagonists was studied in 26 cold-sensitive neurons. Action of baclofen and CGP35348 was studied on 18 and 6 cold-sensitive neurons respectively. Similarly co-application of baclofen and CGP35348 was studied in 2 cold-sensitive neurons. Spontaneous activity was reduced in 16 cold-sensitive neurons during the application of baclofen with a significant difference of $p<0.01$ (Paired t-test). TC increased in 15 cold-sensitive neurons with a significant difference of $p<0.05$ (Paired t-test). CGP 35348 increased firing rate in 6 cold-sensitive neurons and showed a significant difference at $p<0.01$ (Paired t-test). TC values showed a significant difference of $p<0.05$ (Paired t-test) during CGP35348 application (10 μM). Superfusion of both baclofen (1 μM) and CGP35348 (10 μM) did not show significant differences (Paired t-test) either in firing rate or TC values. These results have been further described in detail under the sub heading 'Plasticity of chick hypothalamic neurons: effect of GABAergic substances' in the results part.

Action of a cold-sensitive neuron under superfusion with $GABA_A$ and $GABA_B$ agonist substances during synaptic blockade with calcium free ACSF

In figures 16 (a, b and c), original experimental protocol of the neuronal activity and temperature recorded close to the slice, influence of synaptic block with calcium free ACSF, later during the superfusion of $GABA_A$ and $GABA_B$ receptor agonists namely muscimol and baclofen on a cold -sensitive neuron with calcium free ACSF has been illustrated.

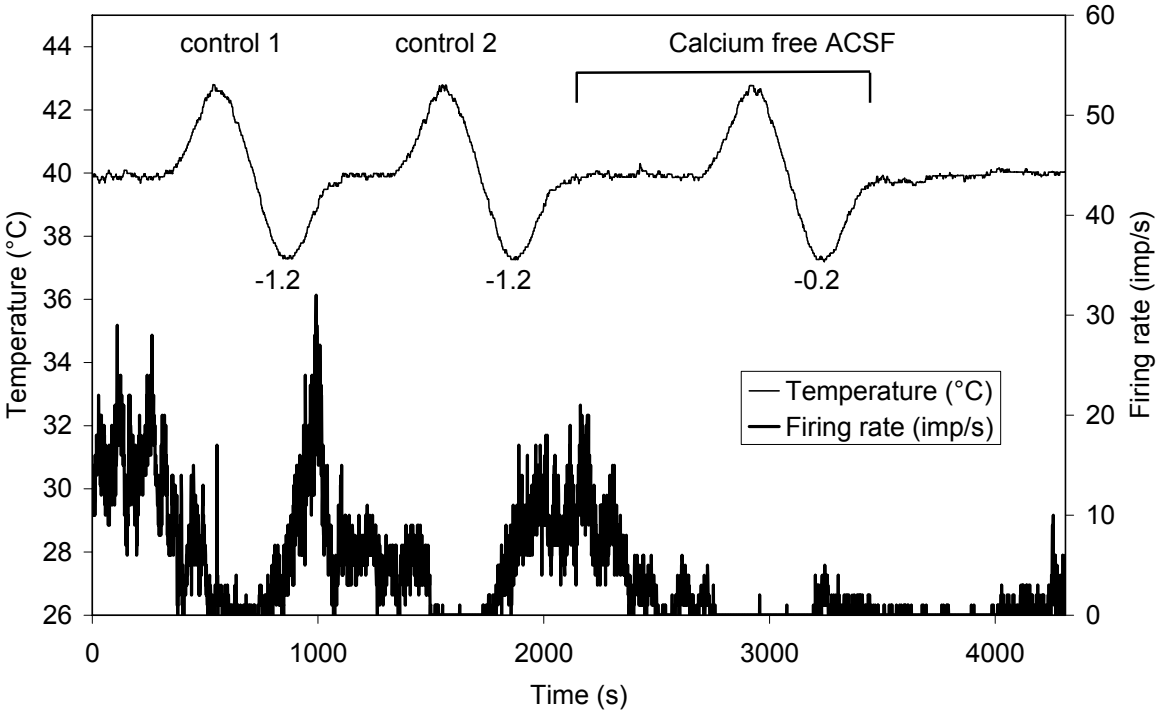

Figure 16(a) : A cold-sensitive hypothalamic neuron under synaptic blockade with calcium free ACSF which exhibits activity during the sinus. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C): two control stimuli, action under calcium free ACSF. Lower panel: Firing rate in imp/s.

In figure 16 (a), the neuron exhibits cold sensitivity during both the control stimuli. The neuron makes a partial recovery during superfusion with calcium free ACSF (synaptic block) exhibiting inherent tendency.

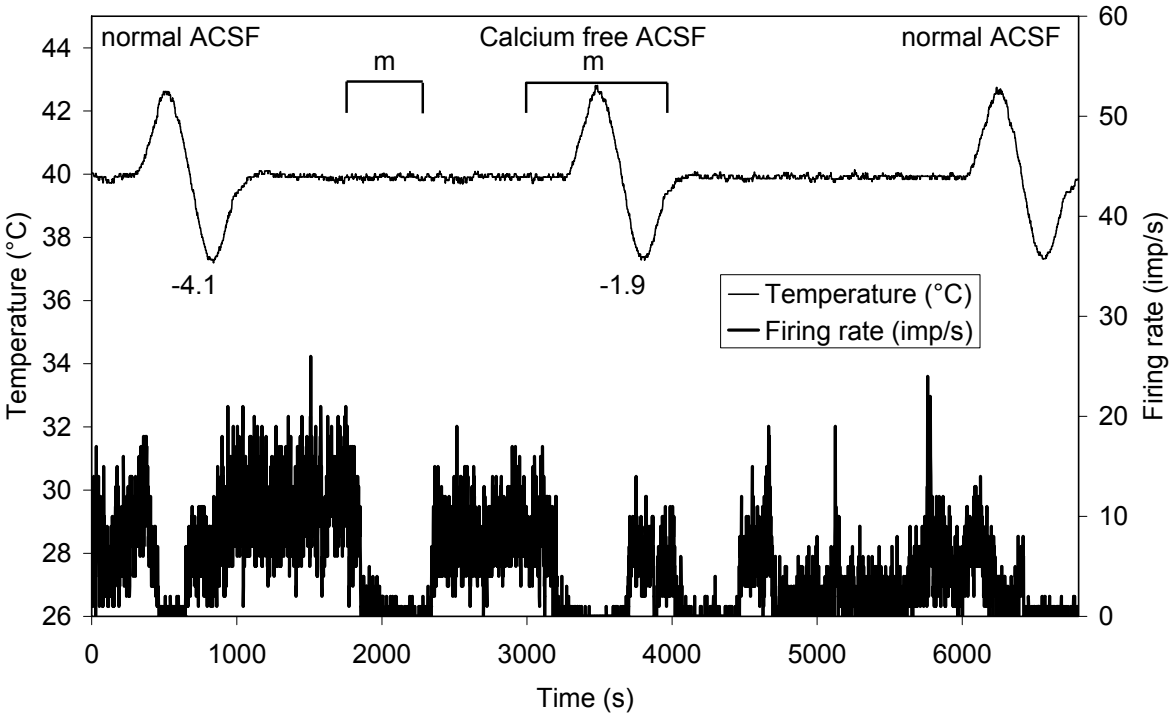

Figure 16(b) : Action of a cold-sensitive neuron under normal ACSF and effect of muscimol under different conditions. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C): one stimulus under normal ACSF, action of muscimol without sinus under normal ACSF and under calcium free ACSF during sinus and a recovery sinus. Lower panel: Firing rate in imp/s.

In figure 16 (b), the neuron shows recovery under normal ACSF after washout and retains its cold sensitivity. Then the neuron is subjected to the action of muscimol during normal ACSF without sinus where it is suppressed. This condition was simulated to ascertain the action of $GABA_A$ agonist under normal circumstances without the influence of temperature on the neuron. Then the neuron is subjected to synaptic block with calcium free ACSF and superfusion under muscimol during the sinus. During this period the neuron exhibits activity and remains cold-sensitive in nature. Later a recovery sinus is made under normal ACSF but the neuron could not recover totally, hence a second recovery is made as shown in the subsequent figure 16 (c).

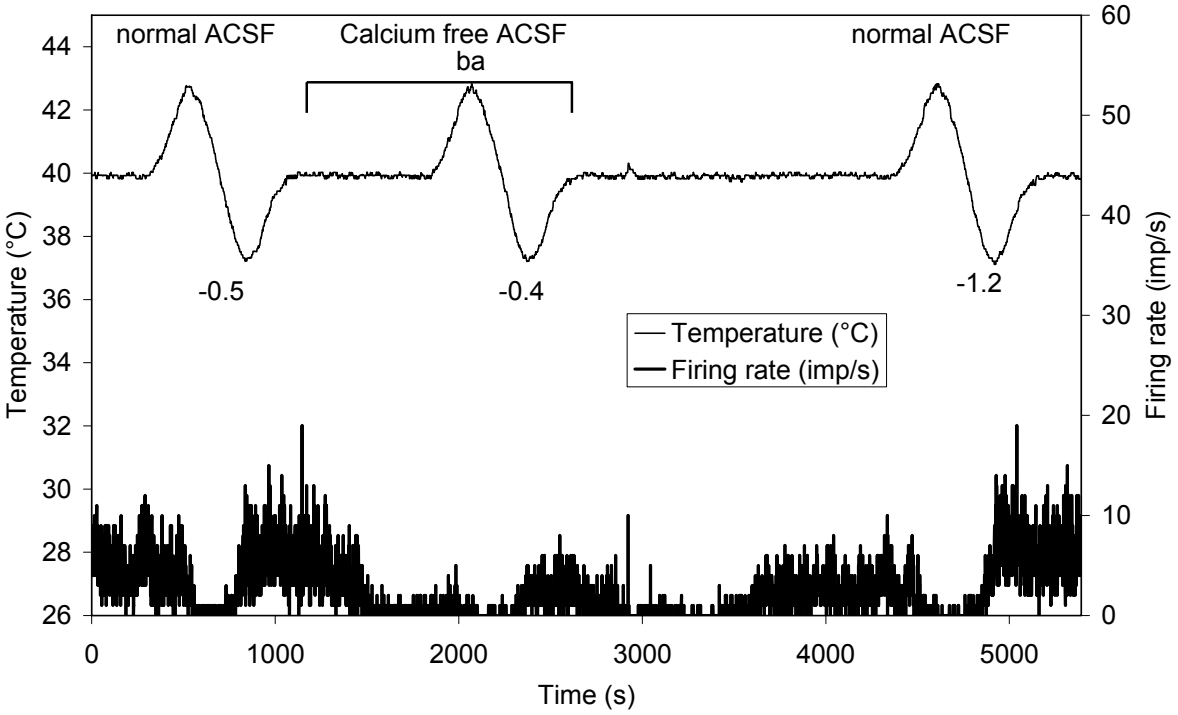

Figure 16(c): A cold-sensitive neuron during sinusoidal temperature stimulation under normal ACSF, under the action of baclofen during calcium free ACSF and a recovery sinus. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C); stimulus under normal ACSF, action of baclofen under calcium free ACSF during sinus and recovery sinus. Lower panel: Firing rate in imp/s.

In figure 16 (c), during the subsequent wash, the neuron recovered and showed cold sensitivity under normal ACSF. As an initial test was already made with $GABA_A$ receptor agonist muscimol under normal ACSF without sinus, another test to ascertain the action of baclofen was not made (and also with previous studies on baclofen's action to inhibit the neuron under normal ACSF, as mentioned under the sub heading number 3.1. – series 1, of the results part as shown in figure 11). Then the neuron is subjected to synaptic block with calcium free ACSF and superfusion under the action of baclofen during the sinus. During this period the neuron exhibits activity and shows cold-sensitivity. After washout, the neuron recovers and exhibited cold sensitivity during the recovery sinus (normal ACSF).

3.3 Plasticity of chick hypothalamic neurons: effect of the GABAergic substances

Two series of experiments have been performed in a probe undertaken to make a comprehensive investigation to find out the mechanism of GABAergic action and the modulation of temperature by its respective agonists and antagonists in 173 neurons.

In the first series of investigations, the effect of the $GABA_B$ receptor agonist baclofen in 35 chick hypothalamic neurons has been studied. In the second series of experiments the effect of synaptic blockade under calcium free ACSF on cold-sensitive neurons and the effect of GABAergic substances has been studied independently as mentioned under their sub headings respectively.

In table 5, a summary of the total number of warm, cold and insensitive neurons investigated in both the series of the experiments to study the effect of GABAergic substances namely the $GABA_A$ and $GABA_B$ receptor agonists and their antagonists has been presented.

Table 5: Total number of neurons investigated during the action of GABAergic substances in both the series of experiments.

Sensitivity of neurons	**Total number of neurons**
Warm sensitive	48
Cold sensitive	56
Insensitive	69
Total number of neurons	173

The study comprises 48 warm-sensitive, 56 cold-sensitive (includes 13 cold-sensitive neurons investigations during synaptic blockade with calcium free ACSF under the sub heading of the results part 'Characterisation of inherently cold-sensitive neurons under synaptic blockade with calcium free ACSF' and action of GABAergic substances) and 69 temperature insensitive neurons. These investigations include the neurons studied under a combination of different substances in a dose dependent manner.

Neuronal investigations made under the influence of $GABA_A$ receptor agonist muscimol and $GABA_B$ receptor agonist baclofen and their respective antagonists bicuculline and CGP35348 have been presented in table 6.

Table 6: Total number of neurons investigated under $GABA_A$ and $GABA_B$ receptor agonists and their respective antagonists independently.

Sensitivity of neurons	$GABA_A$ substances		$GABA_B$ substances	
	Muscimol	**Bicuculline**	**Baclofen**	**CGP 35348**
Warm sensitive	9	6	18	9
Cold sensitive	7	7	18	6
Insensitive	13	11	25	9

3.3.1 Effect of $GABA_B$ receptor agonist baclofen on chicken hypothalamic thermosensitivity: Series 1

In the initial investigations, 35 neurons from the PO/AH were used to study the effect of $GABA_B$ receptor agonist baclofen in the first series of experiments and have been presented adequately in table 7.

Table 7: Total number of neurons under the investigation of $GABA_B$ receptor agonist baclofen.

Sensitivity of neurons	Individual number of neurons
Warm sensitive	11
Cold sensitive	11
Insensitive	13
Total number of neurons	35

Thirteen of the neurons were temperature insensitive (37%), 11 warm-sensitive (31.5%) and 11 cold-sensitive (31.5%). In a total of 35 neurons treated with a concentration of 1 μM of the $GABA_B$ receptor agonist baclofen, 34 (97%) responded with an alteration of tonic activity (firing rate). Only one temperature insensitive neuron did not respond to baclofen superfusion.

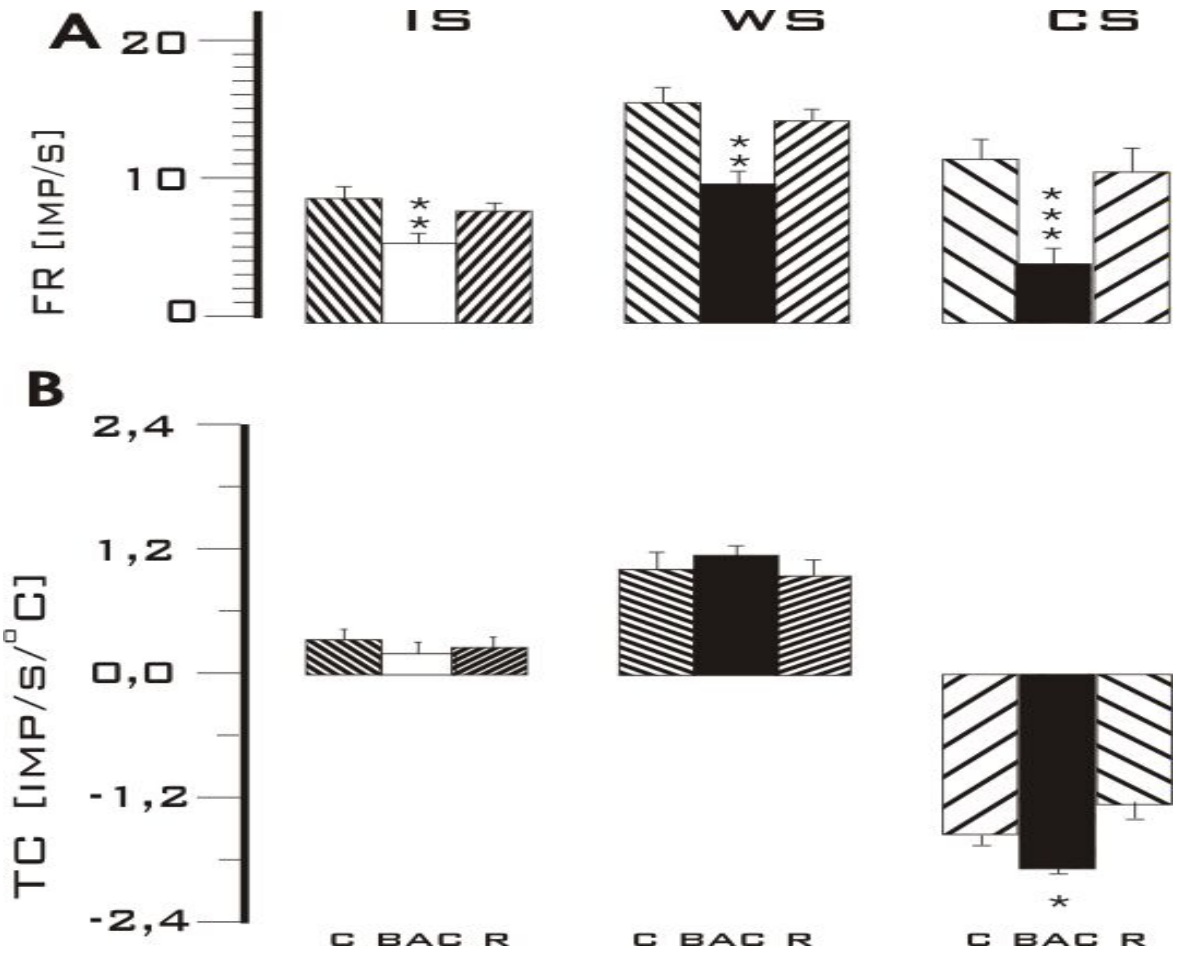

Figure 17: Effects of the $GABA_B$ receptor agonist baclofen (1 µM) on firing rate (FR) (A**) and temperature coefficient (TC) (**B**) of chicken neurons in the PO/AH.**
Data are presented as Δ values (mean Δ values±S.E.M.). Significant differences (paired t-test) between control and baclofen induced effects are indicated: Paired t-test at *p<0.05; **p<0.01; ***p<0.001.
IS – temperature insensitive neurons; WS – warm-sensitive neurons; CS – cold-sensitive neurons.
C – control (before superfusion of baclofen), BAC – effect (during superfusion of baclofen), R – recovery (after wash out of the baclofen). (Data published from a part of the present PhD study: Yakimova *et al*., 2005).

Most of the neurons (30/34) reacted with reduction of firing rate (figure 17 A). Tonic activity was decreased in 82% of the warm-sensitive neurons (9/11) treated with 1 µM baclofen. Nine cold-sensitive neurons (9/11) (82%), as well as 12 temperature-insensitive neurons (100%) were also inhibited at the same drug concentration. One warm-sensitive neuron showed an increased tonic activity and another one has shown biphasic reaction (increasing/decreasing). Firing rate also increased in 2 cold-sensitive neurons treated with baclofen 1 µM.

The tonic activity generally decreased in all types of PO/AH neurons, the temperature sensitivity was changed only in temperature sensitive neurons (Figure 17 B). Trends of increase in temperature coefficient (TC) among the temperature sensitive (warm-sensitive and especially cold-sensitive) neurons was found. The increase of temperature sensitivity

was predominant in cold-sensitive neurons ($p<0.05$; Paired t-test), which were found in high percent (31.5%) in the PO/AH of juvenile chicks investigated. But in some of the neurons (5/11 warm-sensitive and 3/11 cold-sensitive) the TC was decreased. There was no correspondence between the extent of the change in TC and firing rate. Moreover, in some neurons only the firing rate was reduced but not the TC, and in 8 of the 11 temperature-sensitive neurons (2/3 warm-sensitive and 6/8 cold-sensitive) in which the TC was increased, tonic activity was decreased. The TC was not changed in temperature insensitive neurons by baclofen at a concentration of 1 µM.

A typical protocol of the original experiment of a cold-sensitive neuron under baclofen superfusion is given with their respective TC values and recovery is mentioned in figure 18.

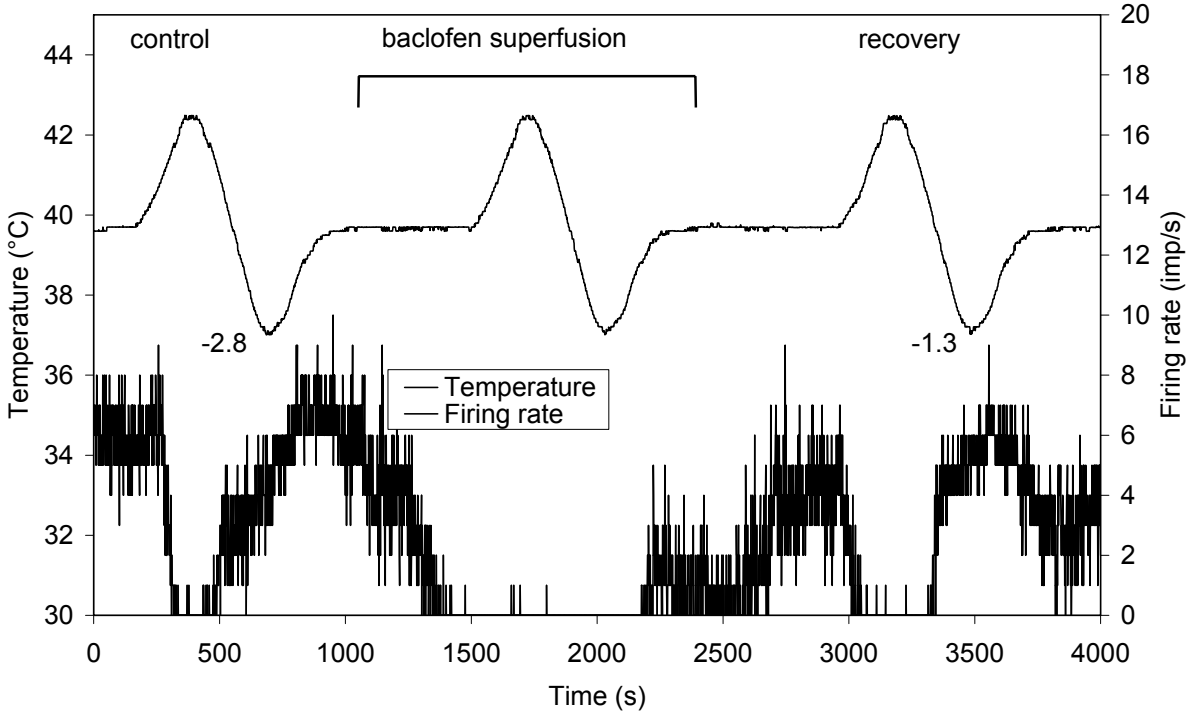

Figure 18: Effect of $GABA_B$ receptor agonist baclofen on a cold-sensitive neuron in preoptic area of the anterior hypothalamus of chicken. Experimental protocol of the neuronal activity and temperature recorded close to the slice. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C); control stimulus, sinus under superfusion of baclofen and a recovery sinus. Lower panel: Firing rate in imp/s.

The neuron under the control stimulus exhibited cold sensitivity. Superfusion of baclofen (1 µM) completely stopped firing rate of the neuron. Later a recovery was observed after washout of baclofen.

3.3.2 A comprehensive study of the effect of the GABAergic substances: Series 2

In the subsequent investigations, to make a comprehensive study of the effect of GABAergic substances inclusive of $GABA_A$ and $GABA_B$ receptor agonists and their respective antagonists, a total of 125 neurons from the PO/AH were used. A similar number of warm-sensitive (37) and cold-sensitive (32) neurons, as well as temperature insensitive (56) neurons were used to compare the effect of GABAergic substances in different populations of chick PO/AH neurons. Detailed investigations performed under synaptic blockade and GABAergic action in relation to cold-sensitive neurons has been described under the sub heading number 2.

3.3.2.1 Effect of substances acting upon $GABA_A$ receptors

Studies made on the action of $GABA_A$ receptor agonist muscimol and its antagonist bicuculline and investigations under the combination of both the substances on PO/AH neurons has been presented in table 8.

Table 8: Total number of neurons investigated under the action of $GABA_A$ receptor agonist muscimol and its antagonist bicuculline and in combination.

Sensitivity of neurons	Muscimol	Bicuculline	Muscimol + Bicuculline
Warm sensitive	9	6	3
Cold sensitive	7	7	3
Insensitive	13	11	4

In figure 19 the influence of muscimol and bicuculline are shown in relation to firing rate and temperature sensitivity under different conditions.

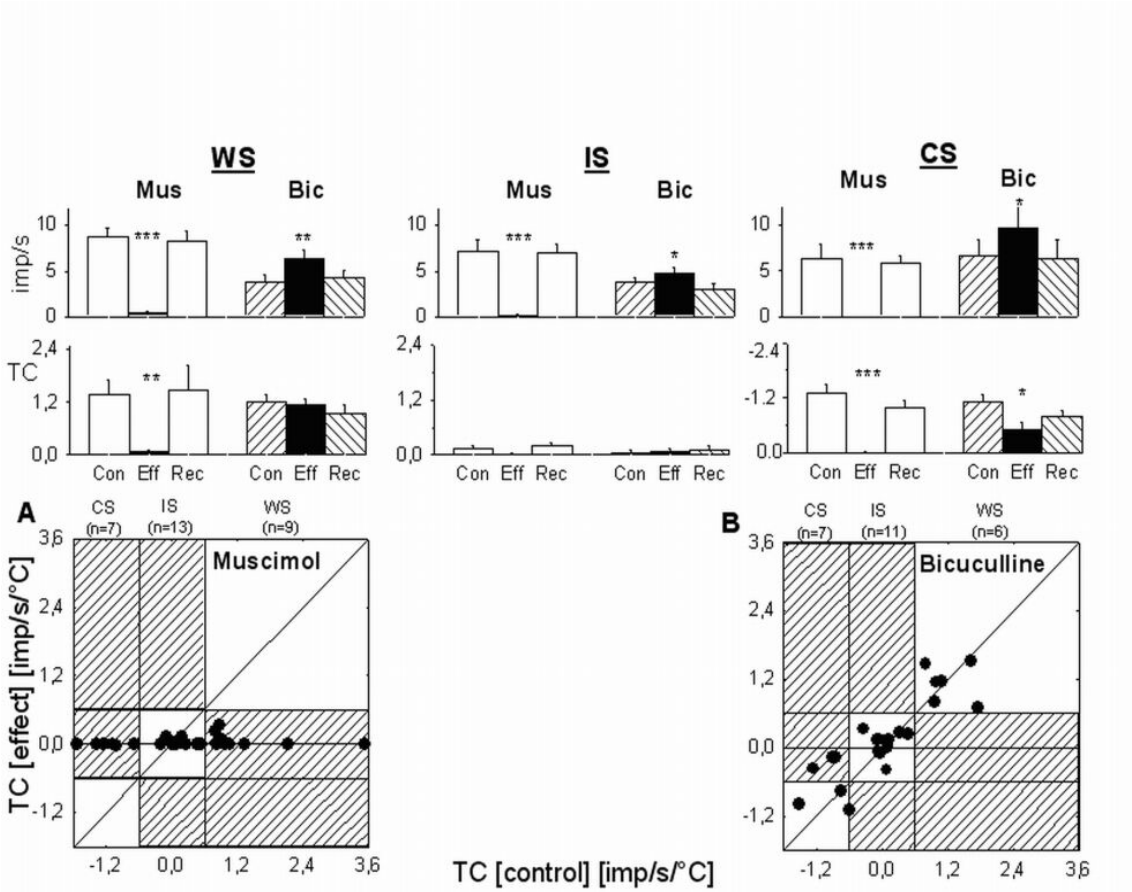

Figure 19: Effect of the $GABA_A$-receptor agonist muscimol and antagonist bicuculline on firing rate and temperature sensitivity of chick PO/AH neurons.
Upper panel: average firing rate (imp/s) and temperature coefficient (TC) of warm-sensitive (WS), temperature-insensitive (IS) and cold-sensitive (CS) PO/AH neurons before (Con), during (Eff) and after (Rec) superfusion with muscimol (Mus) (1 μM) and bicuculline (Bic) (10 μM). Significant values: Paired t-test at *p<0.05; **p<0.01; ***p<0.001; means ±S.E.M.
Lower panel: TC of individual PO/AH neurons during drugs (A, muscimol; B, bicuculline) application [effect] plotted against the TC during the control period before superfusion [control]. Vertical lines define different categories of neurons according to their TC: CS – cold-sensitive; IS – temperature-insensitive; WS – warm-sensitive (n – number of neurons). The distance of a circle from the line of identity indicates the degree of change. Circles in hatched areas represent neurons in which the TC changes were large enough to transform this neuron into another category. Muscimol (1 μM) almost completely inhibited neuronal tonic activity and temperature stimuli were not effective. Bicuculline (10 μM) decreased TC in cold-sensitive neurons and 3 cold-sensitive neurons were transformed into temperature insensitive ones.

Firing rate

Muscimol, an agonist of $GABA_A$ receptors, was used in twenty nine neurons at a concentration of 1 μM. It reduced the spontaneous activity of all neurons regardless of their type of temperature sensitivity (figure 19). The activity of most neurons was reduced to zero and complete recovery was seen after prolonged washing periods (30-45 min) (figure 19 and figure 24).

The $GABA_A$ receptor antagonist bicuculline (10 μM) had the opposite effect on spontaneous activity of chick hypothalamic neurons. Twenty four neurons (6 warm-

sensitive, 7 cold-sensitive and 11 temperature insensitive) were treated with bicuculline. Most of the neurons (20/24) increased the firing rate. Two neurons showed decreased tonic activity and two showed no change in firing rate. Increase in firing rate is statistically significant for all population of neurons ($p<0.01$, Paired t-test), treated with bicuculline, as well as for the different types of neurons separately: warm-sensitive ($p<0.01$, Paired t-test), temperature insensitive ($p<0.05$, Paired t-test) and cold-sensitive ($p<0.05$, Paired t-test) (figure 19). The increase in firing rate was most pronounced in neurons demonstrating low spontaneous activity. The effect of bicuculline outlasted the drug perfusion by 10-15 min.

Temperature sensitivity

In most of the neurons treated with muscimol (1 μM) the temperature stimulus did not have effect and the TC could not be estimated after drug application since the tonic activity was completely inhibited (figure 19 and figure 24). Recovery of the neuronal temperature sensitivity was found after prolonged washing period lasting between 30 to 60 minutes. The $GABA_A$-receptor antagonist bicuculline (10 μM) did not change significantly the temperature sensitivity of warm-sensitive and temperature insensitive chick PO/AH neurons, but significantly decreased TC ($p<0.05$, Paired t-test) in cold-sensitive neurons (figure 19).

In figures 20 and 21 different actions of $GABA_A$ receptor agonist and antagonist has been shown.

In figure 20, the effect of $GABA_A$ receptor agonist muscimol on an insensitive neuron has been shown, where the neuronal activity is totally inhibited during the superfusion of muscimol. Similarly in figure 21, the effect of $GABA_A$ receptor antagonist bicuculline on an insensitive neuron has been shown.

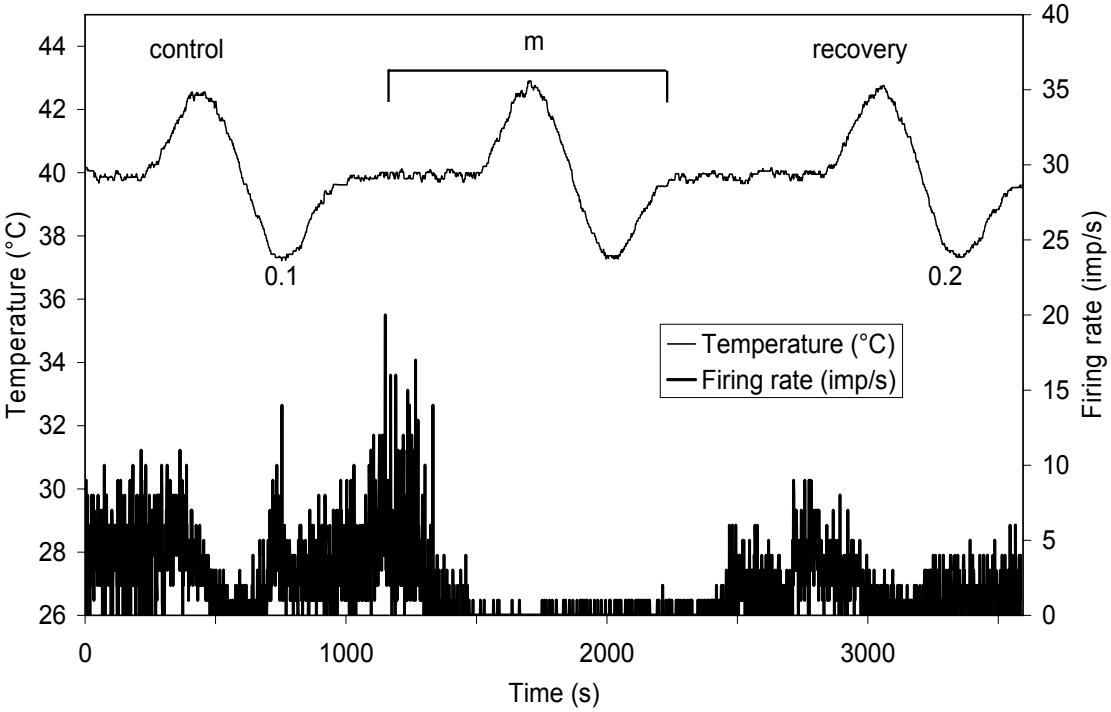

Figure 20: Effect of $GABA_A$ receptor agonist muscimol on an insensitive neuron in preoptic area of the anterior hypothalamus of chicken. Experimental protocol of the neuronal activity and temperature recorded close to the slice. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C); stimulus under normal ACSF, superfusion of muscimol during sinus and a recovery sinus. Lower panel: Firing rate in imp/s.

The action of muscimol on an insensitive neuron decreases the firing rate totally and the temperature coefficient (TC) value could not be evaluated. After washout the neuron recovers and the neuron remains insensitive during the recovery sinus.

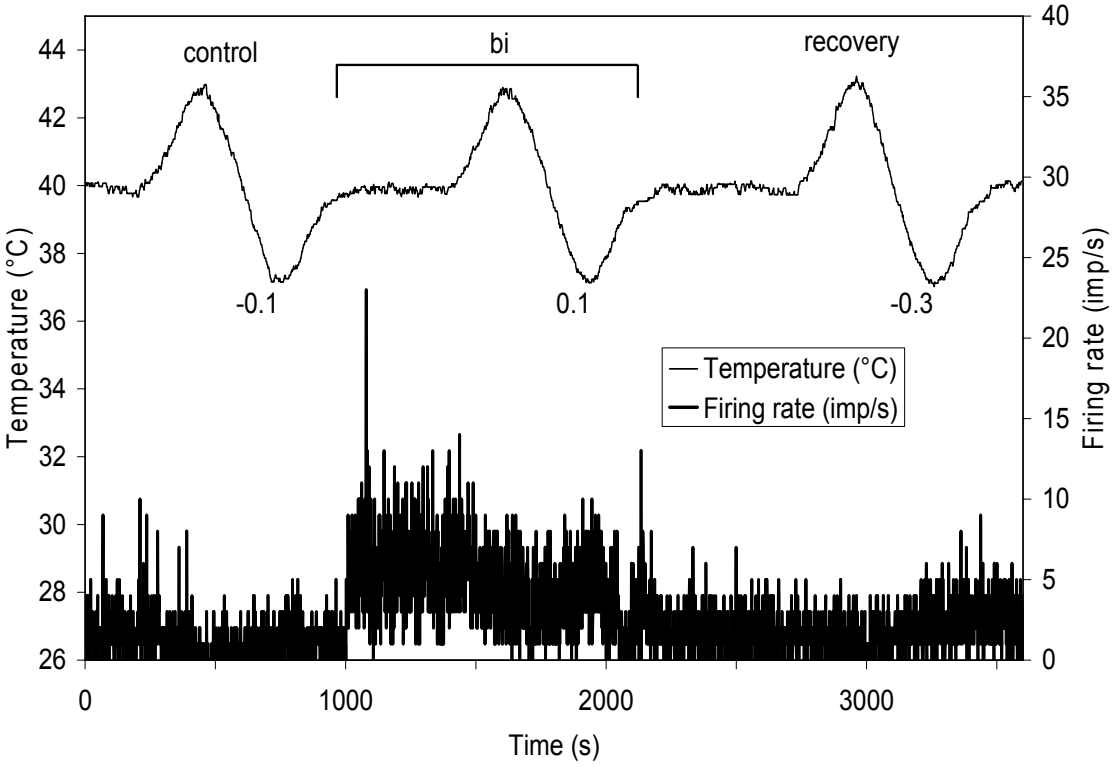

Figure 21: Effect of $GABA_A$ receptor antagonist bicuculline on an insensitive neuron in preoptic area of the anterior hypothalamus of chicken. Experimental protocol of the neuronal activity and temperature recorded close to the slice. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C); stimulus under normal ACSF, superfusion of bicuculline during sinus and a recovery sinus. Lower panel: Firing rate in imp/s.

The neuron under control stimulus was found to be temperature insensitive. Later the action of bicuculline on this insensitive neuron augmented the firing rate and also increased the TC value. After washout the neuron stabilizes and remains insensitive during the recovery sinus.

3.3.2.2 Effect of substances acting upon $GABA_B$ receptors

Studies made on the action of $GABA_B$ receptor agonist baclofen and it antagonist CGP35348 and investigations under the combination of both the substances on PO/AH neurons has been presented in table 9.

Table 9: Total number of neurons investigated under the action of $GABA_B$ receptor agonist baclofen and is antagonist CGP 35348 and in combination.

Sensitivity of neurons	Baclofen	CGP 35348	Baclofen + CGP 35348
Warm sensitive	7	9	3
Cold sensitive	7	6	2
Insensitive	12	9	7

In figure 22 the influence of baclofen and CGP 35348 are shown in relation to firing rate and temperature sensitivity under different conditions.

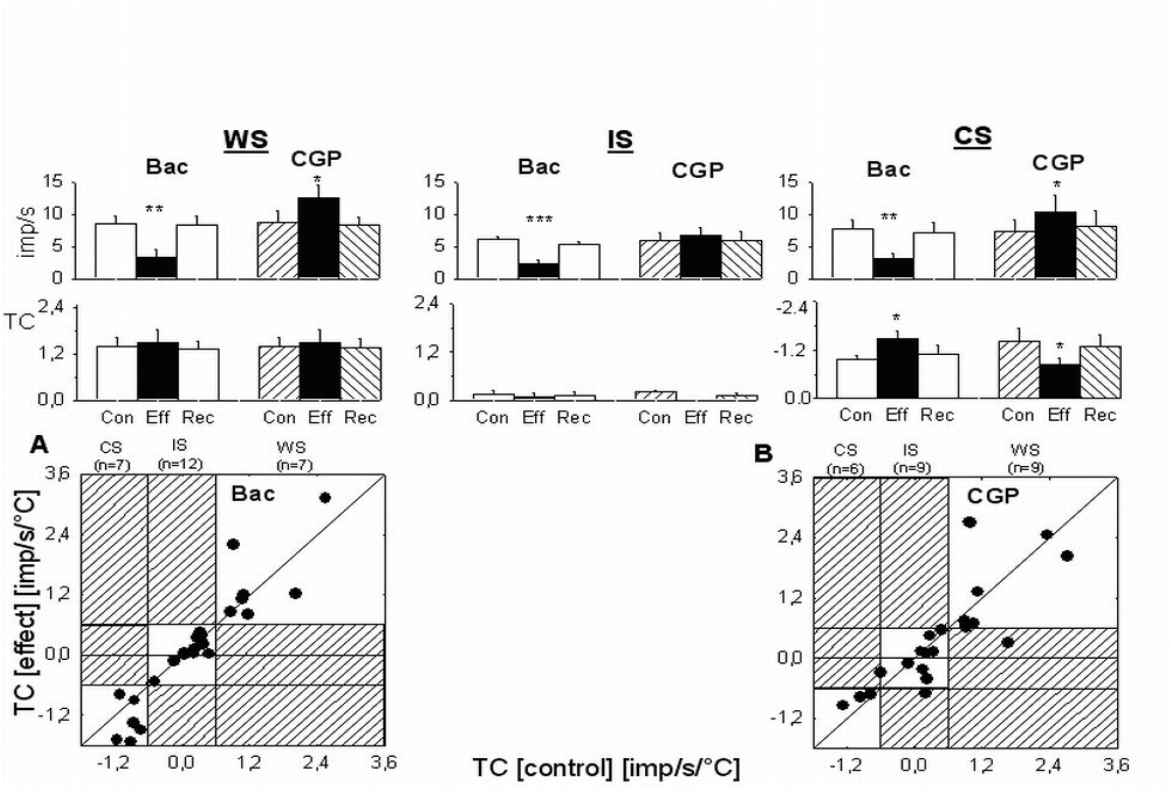

Figure 22 : Effect of the $GABA_B$ receptor agonist and antagonist on firing rate and temperature sensitivity of chick PO/AH neurons.
Upper panel: average firing rate (imp/s) and temperature coefficient (TC) of temperature-insensitive (**IS**), warm-sensitive (**WS**) and cold-sensitive (**CS**) PO/AH neurons before (Con), during (Eff) and after (Rec) superfusion with baclofen (**Bac**) (1 μM) and CGP 35348 (**CGP**) (10 μM). Significant values: Paired t-test at *p<0.05; **p<0.01; ***p<0.001; means ±S.E.M.
Lower panel: TC of individual PO/AH neurons during drugs (**A**, baclofen; **B**, CGP 35348) application [**effect**] plotted against the TC during the control period before superfusion [**control**]. Vertical lines define different categories of neurons according to their TC: CS – cold-sensitive; IS – temperature-insensitive; WS – warm-sensitive (n – number of neurons). The distance of a circle from the line of identity indicates the degree of change. Baclofen (1 μM) increased temperature sensitivity in cold-sensitive neurons, as well as in 2 warm-sensitive neurons; CGP 35348 (10 μM) decreased TC in cold-sensitive neurons, as well as in 2 warm-sensitive neurons.

Firing rate

A total of twenty-six neurons in chick PO/AH were treated with the $GABA_B$ receptor agonist baclofen at a concentration of 1 μM. Firing rate was decreased significantly during baclofen superfusion in 7 warm-sensitive ($p<0.01$; Paired t-test), 7 cold-sensitive ($p<0.01$) (Paired t-test), as well as 12 temperature insensitive neurons ($p<0.001$; Paired t-test) (figure 22). The beginning of restoration after drug administration was stopped, recovery was relatively fast (3-4 min) and complete recovery was observed after 7-8 minutes washing period. On the other hand, the $GABA_B$ receptor antagonist CGP 35348 (10 μM) increased significantly firing rate in twenty four neurons ($p<0.01$; Paired t-test). Firing rate was increased significantly ($p<0.05$; Paired t-test) in 9 warm-sensitive and 6 cold-sensitive neurons, but did not significantly change ($p>0.05$; Paired t-test) in 9 temperature insensitive neurons (figure 22).

Temperature sensitivity

Both the $GABA_B$ receptor agonist as well as the antagonist specifically altered the TC of cold-sensitive neurons, while the TC of warm-sensitive and temperature insensitive neurons was not significantly changed (Paired t-test) (figure 22). The $GABA_B$ receptor agonist baclofen at a concentration of 1 μM increased the TC of cold-sensitive neurons ($p<0.05$; Paired t-test). The specificity of the effect of $GABA_B$ergic substances is confirmed by the finding that the $GABA_B$ receptor antagonist CGP 35348 had the opposite effect on the temperature sensitivity of cold-sensitive chick PO/AH neurons. At a concentration of 10 μM, CGP 35348 decreased significantly the TC ($p<0.05$; Paired t-test). It should be noted that the TC changes induced by baclofen and CGP 35348 were completely reversed after washing with ACSF (figure 22).

3.3.2.3 Effect of simultaneous application of $GABA_A$ and $GABA_B$ receptor agonists and antagonists

The effect of $GABA_A$ receptor agonist muscimol and its antagonist bicuculline has been studied in combination. At the same time $GABA_B$ receptor agonist baclofen and its antagonist CGP 35348 has also been studied in combination .These results are depicted in figures 23 and 24.

Firing rate

Superfusion with bicuculline in tenfold higher concentration completely restored the neuronal activity inhibited by previous application of muscimol and totally prevented the inhibitory action of muscimol applied simultaneously with bicuculline (figure 24 and figure 23 A).

Superfusion with CGP 35348 in tenfold higher concentration completely restored the neuronal activity inhibited by previous application of baclofen and totally prevented the inhibitory action of baclofen applied simultaneously with CGP 35348 on 12 chick PO/AH neurons (3 warm-sensitive, 2 cold-sensitive and 7 temperature insensitive) (figure 23 C).

Temperature sensitivity

No significant change (Paired t-test) of TC was found when muscimol was applied simultaneously with tenfold higher concentration of bicuculline on ten chick PO/AH neurons (3 warm-sensitive, 3 cold-sensitive and 4 temperature insensitive) (figure 23 B).

The baclofen induced increase in TC could be entirely prevented by 10 μM CGP 35348. When CGP 35348 (10 μM) was co-perfused with 1 μM baclofen no significant changes (Paired t-test) in the TC occurred (figure 23 D).

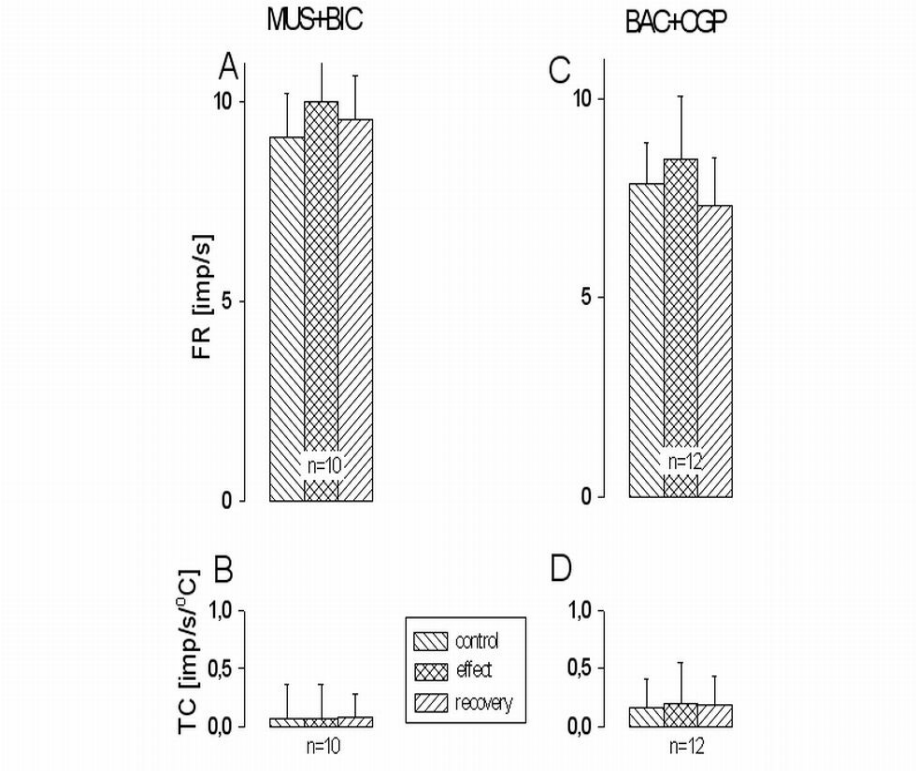

Figure 23: Effect of simultaneous application of GABA receptor agonists and antagonists on firing rate and temperature sensitivity of chick PO/AH neurons.
Superfusion of muscimol (1 μM) and bicuculline (10 μM) (MUS+BIC) indicates firing rate (**A**) and temperature sensitivity (**B**). Superfusion of baclofen (1 μM) and CGP 35348 (10 μM) (BAC+CGP)

indicates firing rate (**C**) and temperature sensitivity (**D**). Data are presented as mean values ± S.E.M.; n – number of neurons.

Figure 24, shows the effect of $GABA_A$ receptor agonist muscimol under temperature stimulus followed by recovery after a prolonged wash out. Specificity of action of individual substances without sinus and with sinus is tested. Later the neuron is subjected to a combination of substances in a dose dependent manner.

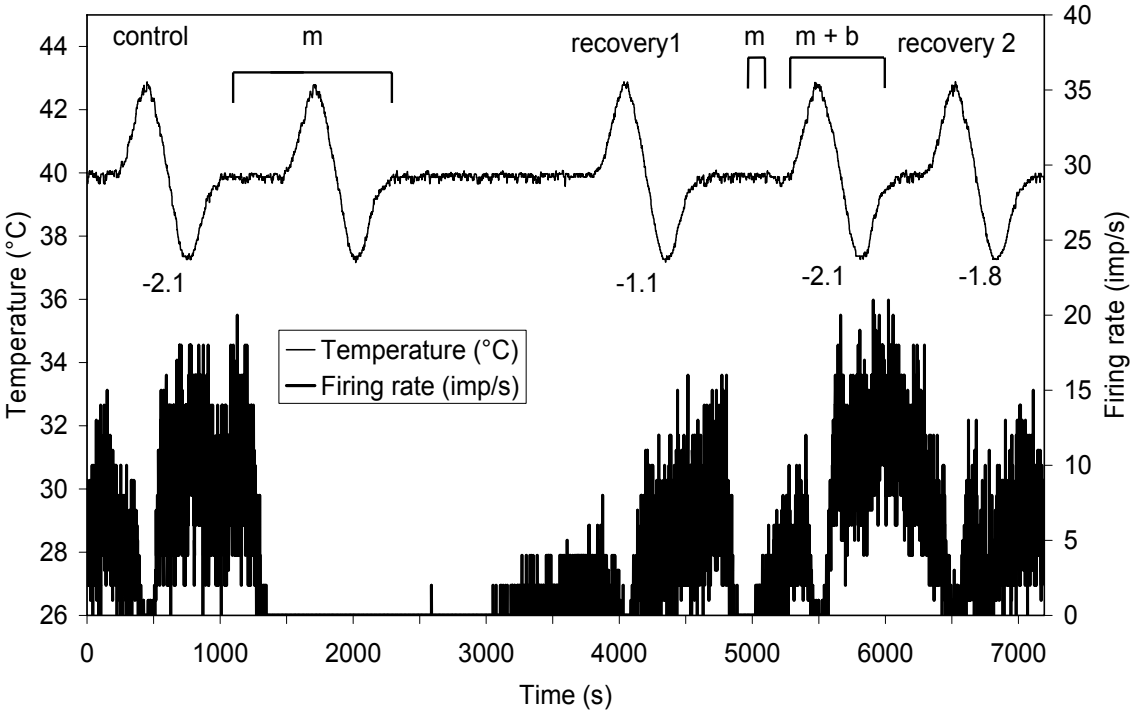

Figure 24: Effect of the $GABA_A$ receptor agonist muscimol and also in combination with its antagonist bicuculline on a cold-sensitive neuron in chick PO/AH. Original experimental protocol of the neuronal activity and temperature recorded close to the slice. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.

Upper panel: Temperature stimulus (°C); stimulus under normal ACSF, superfusion of muscimol during sinus and a recovery, specificity of action of muscimol tested without sinus, action under the combination of muscimol and bicuculline and a recovery sinus. Lower panel: Firing rate in imp/s.

To test the specificity of action of the substance, a control stimulus was made and the neuron was found to be cold-sensitive. Superfusion was made with muscimol (1 μM) which resulted in total inhibition of the neuron and the TC value could not be evaluated during the sinus. To test the effect of muscimol (1 μM), it was applied on the neuron for a brief period without sinus where the firing rate was totally suppressed. Later bicuculline (10 μM) was co-applied with muscimol (1 μM) and the neuron restored its normal activity

remaining cold-sensitive. During recovery the neuron continued to be cold-sensitive in nature.

The effect of $GABA_A$ receptor agonist muscimol and co-application of muscimol with bicuculline has been illustrated in figures 25 (a and b) on a warm-sensitive neuron.

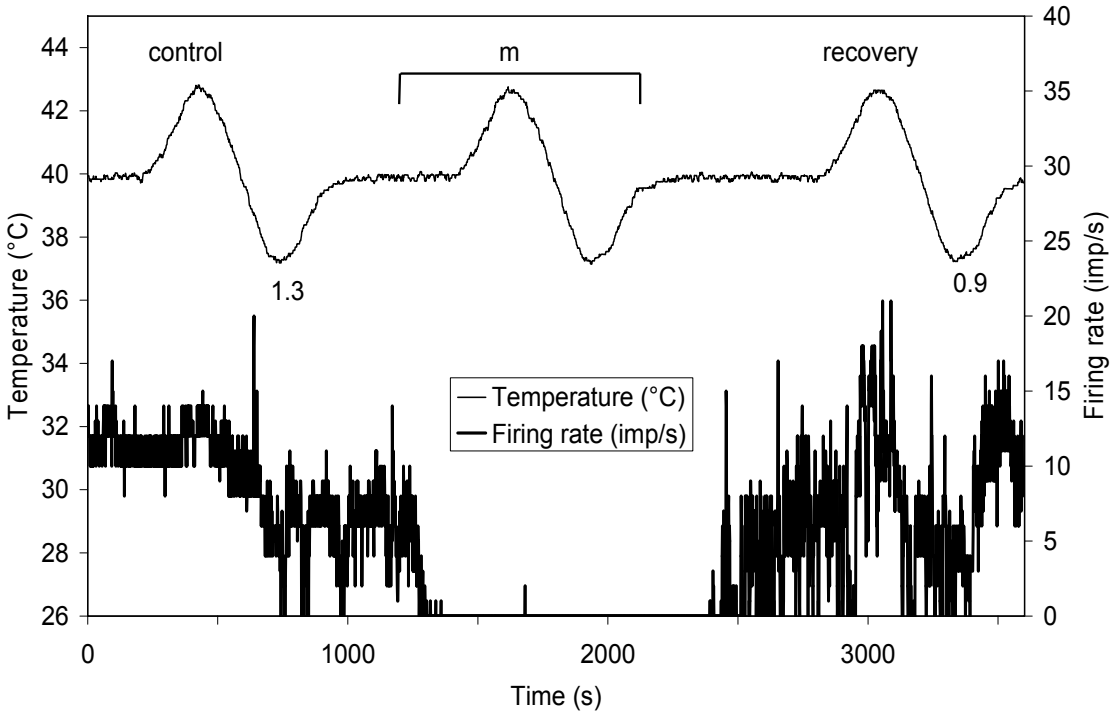

Figure 25(a) : Effect of $GABA_A$ receptor agonist muscimol on a warm-sensitive neuron in preoptic area of the anterior hypothalamus of chicken. Experimental protocol of the neuronal activity and temperature recorded close to the slice. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure are mentioned in the text.
Upper panel: Temperature stimulus (°C); stimulus under normal ACSF, superfusion of muscimol during sinus and a recovery sinus. Lower panel: Firing rate in imp/s.

The action of muscimol (1 μM) on a warm-sensitive neuron causes total inhibition and as a consequence TC value could not be evaluated during the sinus. During recovery, the neuron retains warm sensitivity

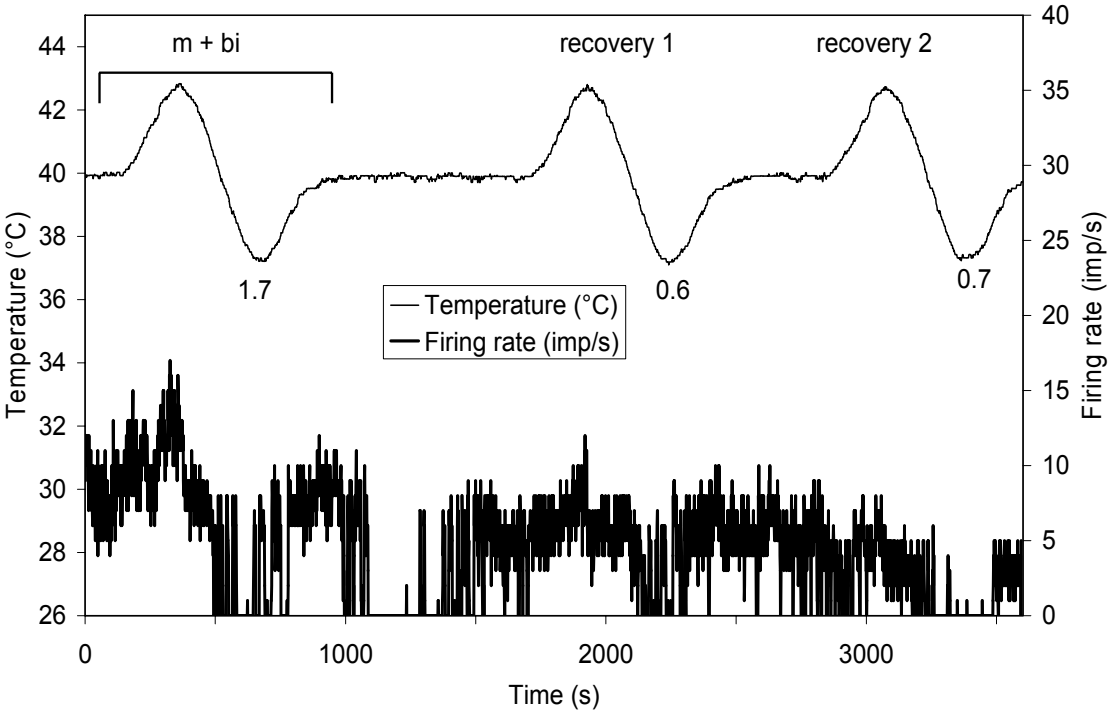

Figure 25(b): Effect of co-application of $GABA_A$ receptor agonist muscimol and bicuculline on a warm-neuron in preoptic area of the anterior hypothalamus of chicken. Experimental protocol of the neuronal activity and temperature recorded close to the slice. The calculated thermal coefficient for a given temperature stimulus is indicated at the responses for each temperature stimulus. Detailed description of the events in the figure is mentioned in the text. Upper panel: Temperature stimulus (°C); stimulus under the action of muscimol and bicuculline and two recovery sinuses. Lower panel: Firing rate in imp/s.

During the co-application of 1 μM muscimol and 10 μM bicuculline the warm sensitivity has been retained. An augmentation in the firing rate and TC values has been observed during the co-application of the respective substances. Later recovery is made two times to ascertain the normal recovery and the neuron remained warm-sensitive in the due course.

4 DISCUSSION

4.1 Neuronal hypothalamic thermosensitivity: early postnatal developmental profile in chicken

In precocial birds during the early postnatal period, the development of neuronal thermoregulatory mechanism, for instance of the neuronal hypothalamic thermosensitivity, is characterized by high plasticity.

Thermosensitivity can be influenced by both exogenous as well as endogenous factors. Among the exogenous factors temperature is one such factor which plays a major role in thermoregulation. And age related changes are also observed during this process of development in relation to thermosensitivity. For instance temperature adaptation in Muscovy duck (Tzschentke and Basta 2002) shows a profound effect with the changes in relation to the proportion of different thermosensitive and insensitive neurons during different age groups. Certain endogenous substances like bombesin show modulatory effect on neuronal thermosensitivity in Muscovy ducks (Tzschentke *et al.,* 2000) and in adult rats (Schmidt *et al.,* 1993).

Changes of the internal milieu results not only from the alterations of the extracellular composition or osmolality but also from changes of the local hypothalamic temperature. In some homoeothermic animals deviations of hypothalamic and body temperature occur under external or internal heat or cold stress (Baker, 1982; Jessen *et al.,* 1992). Consequently, the local temperature of the hypothalamic neuronal controller may change considerably and also the temperature sensitivity of neurons participating in the control circuit might change as it has been demonstrated for different variations of the interior milieu. In this relation the present studies elucidate the role of the hypothalamic neurons and their plasticity with the increase in age.

During the present investigations, a prominent neuronal cold sensitivity has been observed in the investigated age groups of 5, 10, 15 and 20 days but with a difference in 30 days old chicken in relation to the thermosensitive neurons as shown in figure 8. From the results it is obvious that thermosensitivity has shown two different levels of development in the postnatal period in chicken. In the first phase, in the age groups from 5 to 20 days the cold sensitivity shows a gradual increase and this cold sensitivity persists till day 20 and it is very high in this age group (52%) and warm sensitivity (18%) is at the lowest level (table 1).

Significant differences in cold-sensitive and warm-sensitive neurons were found in 15 and 20 days age groups. This could be a sign and a possible reason for a change at this stage of

development which might result in changes in the neuronal network during thermoregulation

This might be a mark (stage) for further changes in thermosensitivity thus clearly exhibiting that majority of the significant differences in neuronal thermosensitivity occurs in these age groups and there occurs a shift later from 20 days onwards. Another aspect in relation to cold-sensitive neurons is that when a comparison is made between 5 days and 10 days; 5 days and 15 days; 5 days and 30 days (tables 2, 3 and 4) exhibited no significant differences but a significant difference was exhibited between 5 days and 20 days. Thus there occurs two stages of development; the first stage could be from day 5 to day 20 where the main characteristic features are an increase in cold sensitivity and with no major differences in warm sensitivity. And the second stage might occur between 20 and 30 days age groups and this kind of development might continue thereafter.

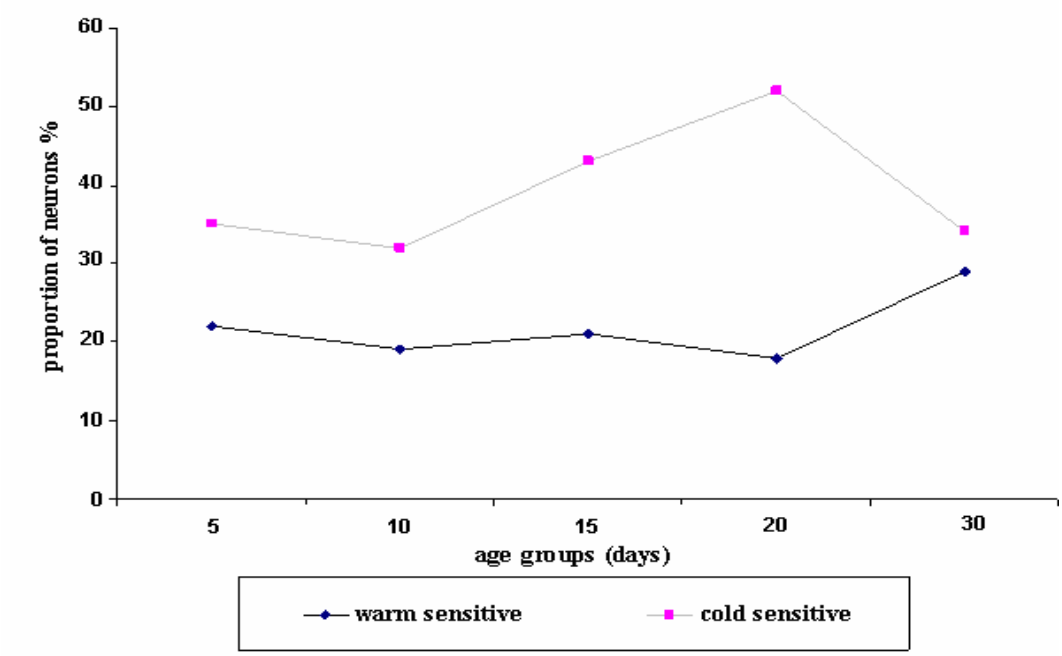

Figure 26: Comparison of warm and cold-sensitive neurons in different age groups in chicken.

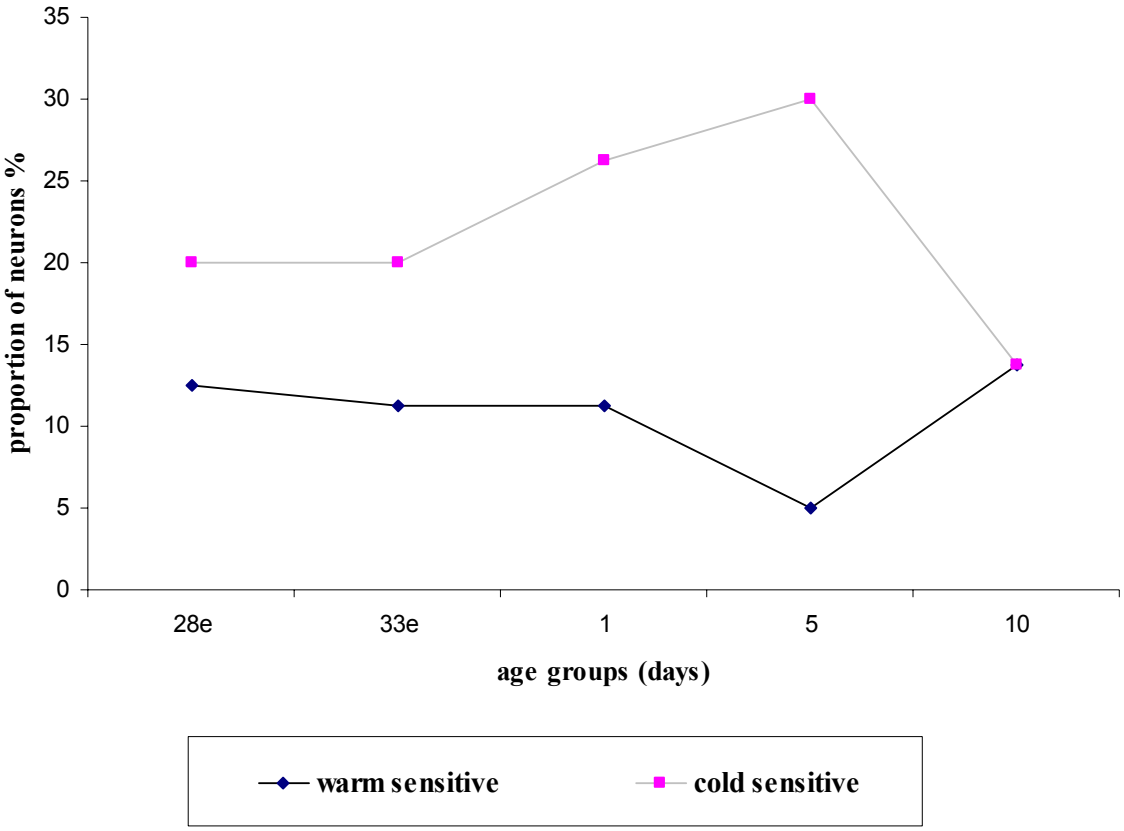

Figure 27: Comparison of warm and cold-sensitive neurons in different age groups in Muscovy ducks (Tzschentke and Basta 2002).

And a similar kind of development could be observed in the Muscovy duck but the differentiation of thermosensitivity being different (figure 27) as described in the ensuing discussion. This kind of developmental pattern has been studied in relation to the Muscovy duck (Tzschentke and Basta 2002) which enables us to understand the species specific differences in the neuronal thermosensitivity in chicken.

As observed from the two stages of development of neuronal hypothalamic plasticity in chicken (figure 26), the neuronal hypothalamic thermosensitivity in Muscovy ducks (Tzschentke and Basta 2000) (figure 27) has been shown to develop in two stages: (1) between day 28 of incubation and day 5 of post hatching, neuronal hypothalamic cold sensitivity increased from 20 to 30% whereas the proportion of warm-sensitive neurons is low and decreased moderately from 12.5 to 5 % in relation to all neurons investigated and (2) between days 5 and 10 of post-hatching, neuronal cold sensitivity of the PO/AH decreased significantly to 14% whereas neuronal warm sensitivity increased significantly to 15%. However, in the Muscovy duck between days 5 and 10 after hatch, a qualitative change occurs in the development of neuronal hypothalamic thermosensitivity from the

"juvenile" to the "adult" type. While, in adult Pekin ducks thermosensitivity of the PO/AH in brain slices is characterised by a low cold sensitivity (6.2%) and a high warm sensitivity (58.3%) (Nakashima *et al.,* 1987), which is similar to adult mammals (Boulant *et al.,* 1989). Similar high neuronal hypothalamic cold sensitivity during early ontogeny was also observed in prenatal temperature experienced Muscovy ducklings (Tzschentke and Basta, 2002). But this qualitative change from the ″juvenile″ to the ″adult″ type occurred in prenatal temperature experienced birds at an earlier stage of development (between day 1 and 5 of post hatching). Furthermore, this earlier change exclusively arose in the cold sensitive neurons (Tzschentke *et al.,* 2004). Thus in the actual study in chickens the first step of the development of neuronal hypothalamic thermosensitivity, which is characterised by high and as well increasing neuronal cold sensitivity occurs during a later developmental period. When a comparison was made between the results of chicken and Muscovy ducks, there occurs a shift of hypothalamic thermosensitivity, which represents species specificity and is obviously related to the different developmental patterns in both species. Altogether, *Galliformes* during early postnatal development are characterised by a lower precocial status than *Anseriformes* (McNabb and Olson, 1996). When a comparative study is made between hatchlings of chicken and Muscovy ducks, a higher heat production capacity in Muscovy ducklings was observed, which enables them to stabilise the body temperature in a narrow temperature range (Tzschentke and Nichelmann, 1999). Because all effector activity is controlled by the nervous system, the earlier maturity of the duck brain corresponds with the developmental status of peripheral mechanisms.

Such a developmental pattern could be as a consequence where different mechanisms of thermoregulatory effector systems develop in the early postnatal period and accordingly changes in the hypothalamic thermosensitivity also occur. These changes are important for the survival of the animal and its ability to adapt to the actual environment. Most of the changes within the peripheral thermoregulatory mechanism also occur in the early postnatal period (Nichelmann and Tzschentke 2003) and this might also contribute for changes in central neuronal thermosensitivity as well.

Differences within the relative frequency of the TC of all PO/AH neurons (during all temperature stimulations) investigated between 5 days and 10 days; 5 days and 15 days; 5 days and 20 days and finally between 5 days and 30 days were not significant (U- test at $p<0.05$) and did not show normal distribution (as shown in figures 9 to 13). In chicken the neuronal network appears to be developed at a later stage. In Muscovy duck, it did not exhibit normal distribution in the early stages and later the normal distribution could be

seen with a significant difference on day 10 post hatching (Tzschentke and Basta 2002). This kind of distribution in Muscovy duck lead to a characterization of improved neuronal network on day 10 post hatching when compared to the young ones. There occurs a change from juvenile to the adult on day 10 post hatching in Muscovy ducks. But in chicken this kind of neuronal maturity could be observed only at a later stage on day 30 post hatching (figures 26 and 27). Hence the relative frequency of the TC of the PO/AH neurons (during all stimulations) for characterization of maturation during the development in chicken could not be used as a parameter as suggested in Muscovy duck (Tzschentke and Basta 2002).

Developmental pattern in relation to peripheral mechanisms

The present developmental pattern in chicken could be discussed in the light of various physiological and behavioural patterns observed in different species both in the late embryonic and early postnatal periods.

To explain the neural control of the body temperature, several models suggest that preoptic warm-sensitive neurons may also inhibit and cold-sensitive neurons may enhance heat production (shivering and non-shivering thermogenesis) and heat retention (cutaneous vasoconstriction and thermoregulatory behaviour). Body temperature is therefore maintained constant by the balance between heat loss responses and heat production/retention responses. A shift in this balance towards the later responses results in an increase in body temperature. In the ensuing phenomena heat production and heat retention processes might have an impact on the maturity of the developing system especially in relation to the physiological processes. During early postnatal development physiological mechanisms are not completely mature, for instance the heat production capacity is low during this period. On the other hand thermoregulatory behaviour is a highly effective mechanism to keep body temperature constant. In this regard the poultry hatchlings are able to select their specific temperature gradient with high accuracy immediately after hatching. During this period behavioural thermoregulatory mechanism, such as the innate ability to prefer ambient temperature are essential for maintenance of homeothermy because the autonomic mechanisms of thermoregulation are not fully developed (Nichelmann and Tzschentke 2003). Compared to physiological regulation of body temperature, behaviour is a phylogenetically older but very effective means of thermoregulation (Kluger 1979). Homeothermic organisms attain effective thermoregulation with minimal disruption of other homeostatic system by physiological

(e.g. heat production, heat loss, vasoconstriction or vasodialtion) as well as behavioural (e.g. social thermoregulation, temperature preference) mechanism (Schmidt 1976). Interactions between behavioural and physiological thermoregulatory mechanisms reduce high cost energy of thermoregulatory heat production, which is necessary for maintenance of stable body temperature, to a large degree.

Peripheral thermoregulatory mechanisms are rather ineffective before hatch as shown in various investigations on different precocial bird species. Endothermic reactions occur but only with in a very limited range in fowl and duck embryos (Nichelmann *et al.,* 1998; Nichelmann and Tzschentke 1999). In comparison to this, the efficiency of heat loss mechanisms, such as blood flow changes in the chorio-allantoic membrane and respiratory evaporative loss is much higher at an earlier stage (Nichelmann and Tzschentke 2003). Invstigations by Janke *et al.* (2002) on embryonic development of heat production revealed a strong relationship between body core temperature (Tc) and oxygen consumption which could be described by a highly significant linear regression. An increasing body core temperature with increasing oxygen consumption occurs when nervous thermoregulatory mechanisms and effective thermoregulatory control elements are not functional as well developed control system with high efficiency would allow a well balanced equilibrium between heat loss and heat production (HP) and adjust the core temperature to the thermoregulatory set point (Janke *et al.,* 2002). Although at first an effective thermoregulation is not yet required for the survival of the embryo nevertheless it is important that endothermic reactions occur (Nichelmann *et al.,* 1998a; 1998b) to ensure the complete development of body functions and as well thermoregulatory system shortly after hatch (Nichelmann and Tzschentke 2003). At the same time on the first day of life when heat production (HP) increases and thermoregulatory elements like evaporative heat loss are developed, the animal can maintain a constant body temperature over fairly wide range of temperatures.

On the first day of life all thermoregulatory mechanisms are working (which had already started functioning in prenatal period). But the different thermoregulatory mechanisms *viz.,* behavioural and autonomic the constituents of the peripheral mechanisms have different levels of maturity after hatching. The autonomic mechanisms are mature late, but the innate behaviour shows higher activity immediately after hatch. Hence the birds might use the behavioural mechanisms to keep the body temperature constant. As a result a feed back mechanism exists wherein an interaction between the peripheral and central mechanisms occurs influencing each other.

Changes in Ta and Tc lead to an activation of thermoregulatory control elements in a typical order and with in the first ten days of life the development of the thermoregulatory control system seems to be complete.

It was also supposed that shortly after hatching birds might have a lower thermoregulatory setpoint, increasing with increasing age. This may be an energy saving mechanism by reducing the thermal gradient between the animal and its surroundings (Hissa *et al.,* 1983). Deep body temperature increases from 39 °C in duck embryos to 40 °C in ducklings (Holland 1998) where as the biological optimum temperature (Nichelmann 1983) and the preferred ambient temperature decrease (Tzschentke and Nichelmann 1999). As such development of peripheral mechanisms during the early postnatal periods could also play a role in the development pattern.

Thus in chicken a qualitative change might occur in the development of neuronal hypothalamic thermosesnitivity from the "juvenile" to the "adult" type as differentiated between the 20 days old age group and 30 days old age group. However, in the Muscovy duck between days 5 and 10 after hatch, a qualitative change occurs in the development of neuronal hypothalamic thermosensitivity from the "juvenile" to the "adult" type. Though the data is scarce in relation to the juvenile forms in the mammals, but the studies conducted in the adults of birds and mammals represent a low cold sensitivity and high warm sensitivity in contrast to the juvenile birds. These observations clearly enunciate a similar pattern of development but the maturity of development occurs at a different time periods in different kinds of species. All these aspects together indicate that the neuronal control of body temperature may vary under the influence of local temperature changes, demonstrating the plasticity of the control system especially in relation to the age related development.

Developmental pattern in relation to synaptic plasticity

In the present investigations in the early postnatal stage, pronounced cold-sensitivity was seen and especially in 20 days age group highest proportion of cold-sensitive neurons (52%) as observed in table 1 was found. And in the second stage after 20 days a decline in the cold sensitivity is observed which continues thereafter. Another mechanism associated with the developmental patterns could be the neuronal mechanisms related to synaptic plasticity.

It is understood that during early ontogenesis functional neuronal circuits develop in two phases, which are triggered by sensory input. In the first phase the number of synaptic

contacts increases, where as in the second phase (synaptic reorganisation) their number decreases (Brown *et al.,* 2004). Only those contacts sustain which are necessary, for instance, to maintain homeostasis or to process emotional signals under the respective surroundings which repeatedly receive sensoric inputs (Bock *et al.,* 2003). Altogether increase in the number of synaptic contacts during the first phase as well as their reduction in the second phase needs sensory (social /environmental) stimulation. Bock and Braun (1999 b) have shown that in comparison to 7 day old social deprived domestic chicks, 'filial imprinting' induced synaptic pruning in higher associative forebrains.

For instance 'Imprinting' describes a fundamental process of life occurring during circumscribed time windows of prenatal and postnatal ontogenesis and having lasting effects (Tzschentke and Plagemann 2006). It explains the determination of social binding as well as physiological control systems related to the environment of developing organism. Konrad Lorenz analysed the development of social binding applying the term 'imprinting' to describe the process. One of his major ideas was that imprinting occurs during 'critical periods', which are limited to and severely restricted to animal's early life (Lorenz, 1935). Learning processes, including emotional experiences during early development induces long-term changes in the pattern of synaptic contacts in respective brain areas (Bock and Braun, 1998). A lack of these processes, e.g. by social deprivation, has long-lasting consequences on formation, function and interaction of synapses with in the functional neuronal networks (Bock and Braun, 1999 a; Ovtscharoff and Braun 2001; Bock *et al.,* 2005 a).

If the sensoric input does not occur then the two steps where synaptic contacts are developed, where, in the first phase an increase in the number of synaptic contacts would not occur which obviously will not result in the reduction of these contacts as a consequence disturbing the homeostasis. This shows the importance of sensoric inputs and the learning process for thermoregulation has to be mature with increasing age. Hence these two steps of development in relation to synaptic contacts could exist where the sensoric inputs play an important part in the developmental process. The increase and as well as the decrease in cold-sensitive neurons could be as a consequence of synaptic induction which could be based on a similar mechanism as explained by Brown *et al.* (2004). Due to this peripheral cold signals could lead to a higher proportion of cold-sensitive neurons within the hypothalamus of the precocial birds in contrast to the adult birds as there has been a decline of cold-sensitive neurons in the later stages of development as shown in the figure 26. The present study in birds might also represent

similar stages (two levels) of development as observed from the results of Brown *et al.,* (2004).

4.1.1 Comparison of neuronal hypothalamic thermosensitivity between juvenile and adult birds and adult mammals

Different patterns of thermosensitivity exist when a comparison is made among the chicken (figure 8), the Muscovy ducks (figure 27) and the mammals (Figure 28).

In all the investigations in mammals, the proportion of warm-sensitive neurons exceeded the number of cold sensitive neurons. This is in contrast to the findings in the hypothalamic thermosensitvity in juvenile precocial birds as well as bird embryos where a high neuronal cold sensitivity is prominent.

The information is scarce about hypothalamic thermosensitivity in juvenile mammals and no literature on mammalian embryos. In order to find differences within the proportion of thermosensitive neurons between juvenile birds and juvenile mammals further investigations are required. In accordance to the fact that metabolic cold defence in birds is different to that of mammals under natural conditions of cold stress (Simon *et al.,* 1986) any differences in neuronal thermosensitivity might contribute to the hypothesis, that central nervous thermosensitivity might have followed different lines of evolution in mammals and birds. But a significant similarity is found among different bird species.

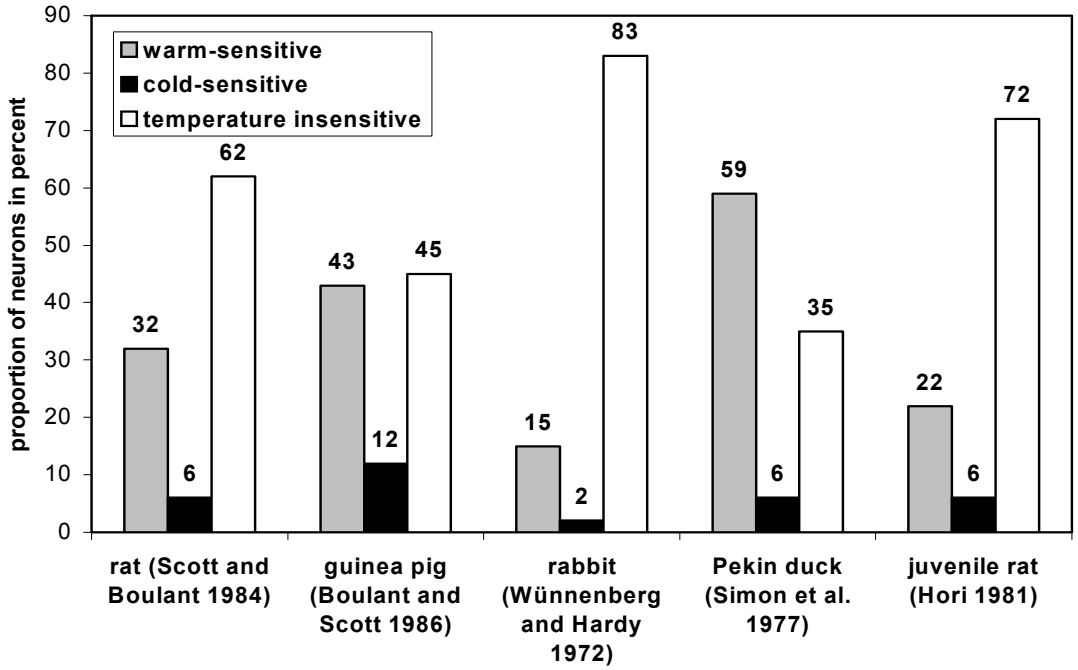

Figure 28: Proportion of cold, warm as well as temperature insensitive PO/AH neurons in different animal species.

During the present investigations, when a comparison is made specifically among the chickens and Muscovy ducks, the cold sensitivity is highest in the 20 days old age groups in chicken and declined in the 30 days old age group. In case of Muscovy duck, cold sensitivity was exhibited very early *viz.,* on day 5 and later declined in post hatch birds of 10 days. A shift towards warm sensitivity occurs very early in the Muscovy duck but in chicken it is prolonged to a later stage of development. During a comparison between the juvenile chicken and Muscovy duck with adult Pekin duck, relatively less cold sensitivity has been observed in the adult Pekin ducks (figure 28). When the cold sensitivity of chicken, and Muscovy duck was compared with the mammalian species, there is a strong decline of cold sensitivity in all the mentioned mammalian species.

The results from the precocial birds namely Muscovy ducks (Tzschentke and Basta 2000) and the chicken are in contrast to various investigations on the hypothalamic thermosesnitivity in adult birds and adult mammals (figure 28). These results suggest that there occurs a change in the developmental pattern from the juvenile to the adult status. But a similar pattern of development could be visualized when a comparison was made with the juveniles of chicken and Muscovy duck in relation to thermosensitive neurons. It would be interesting to compare whether a similar pattern could be established in relation to the juvenile mammals as well. Perhaps this necessitates further investigations in juvenile mammals to make a comprehensive comparison among these forms.

Although in all of these investigations large differences in the distribution of thermosensitive and insensitive neurons occurred, this might be also due to the fact that investigation methods were not standardised within these first experiments. Different limitation values of TC as well as temperature stimuli in differing ranges were used. Furthermore thermosensitivity was studied both *in vivo* and also *in vitro* in different species of birds and also mammals which also might be a cause for the variation of neuronal thermosensitivity.

4.1.2 Thermoregulatory relevance of a high hypothalamic cold-sensitivity in precocial bird species

Few instances of high proportion of cold-sensitive neurons are found in literature but this kind of sensitivity is prominent among the birds, previously found in Muscovy ducks (Tzschentke and Basta 2000) and presently in the chicken. The cold sensitivity occurs in the Muscovy ducks in the early stages of development but in chicken it is observed at a later stage. Hence existence of inherent nature of cold-sensitive neurons, which can act as

thermosensors in the brain, was a question for investigation. As such synaptic blockade of cold-sensitive neurons were performed using calcium free ACSF in view of various arguments where it has been proposed that the influence of calcium might effect the thermosensitivity of a neuron.

A study was made to asses the usefulness of synaptic blocking medium containing low calcium and high magnesium for the differentiation between inherent and synaptically induced temperature sensitivity because even relatively small changes in calcium causes changes in firing rate, temperature coefficient, discharge pattern and action potential amplitude (Schmid and Pierau 1993). There is a strong dependence of temperature sensitivity of neurons on the extracellular calcium; this observation also suggests caution when quantifications of temperature sensitive PO/AH neurons from different labs are compared because the calcium of the normal control ACSF varies from 0.9 to 2.4 mM (Schmid and Pierau 1993). In various preparations the reduction of extracellular calcium results in a concentration dependent increase in firing rate (Richards and Serecombe 1970). Studies measuring the free calcium of different brain areas reported values between 0.75 and 1.5 mM (Heinamann *et al.,* 1977). Solutions containing calcium concentrations of 2.0 mM or more are nonphysiological (Blatteis 1981). In addition to elevated FR, an increase in TC of PO/AH cells in low calcium solution was found (Schmid and Pierau 1993). Superfusing neurons with calcium of <0.7 mM, with one exception caused a significant increase in the TC of all cells investigated. Schmid and Pierau (1993) suggest that temperature sensitivity is not an unchangeable property of a certain population of PO/AH neurons but that it can be altered by small changes in the extracellular calcium.

In adult mammals synaptic blockade shows that most synaptically driven warm-sensitive neurons of the PO/AH are inherently thermosensitive, the inherent thermosensitivity of cold-sensitive PO/AH neurons discussed is controversial (Boulant and Dean, 1986). One hypothesis is that cold-sensitive neurons in the hypothalamus can be viewed as interneurons inhibited by nearby warm-sensitive neurons (Boulant *et al.,* 1989). On the other side, within the low number of cold-sensitive neurons, which can be found in the mammalian as well as the hypothalamus of adult birds, single inherent cold-sensitive neurons were found in brain slices during blockade with Ca^{++}-free/high-Mg^{++} -medium using extracellular recordings (Nakashima *et al.,* 1987). In another study using Ca^{++}-imaging and extracellular cell-attached patch recording primary cold-sensitive neurons with low threshold temperature were found in PO/AH of rats (Abe *et al.,* 2003).

In the actual study, 56 cold-sensitive neurons have been studied under different conditions of synaptic block and also during GABAergic action (table 5). In these investigations, 13 cold-sensitive neurons of 10- to 20 days old chickens were investigated under synaptic blockade with Calcium free ACSF. Within the investigated cold-sensitive neurons, 6 increase the firing rate and four of them strongly, under cold load during synaptic blockage, thus exhibiting an inherently cold sensitivity. And 7 neurons were totally blocked under synaptic blockage which exhibit synaptic induced cold sensitivity. All the neurons showed recovery after wash out subsequently. Some cases of synaptically-induced modulations of neuronal cold sensitivity were observed earlier in the PO/AH slices of adult ducks (Nakashima *et al.,* 1987).

As shown in figure 14, under two control stimuli, neuron is cold-sensitive in nature. During superfusion with calcium free ACSF, the neuronal activity is inhibited. This state of inactivity of neuron persists during the whole of warm phase. But the neuron exhibits activity in the cold phase during a short interval of time. This activity has been observed at the lowest temperature of the cold phase of the sinus. This inherent tendency of the neuron which shows activity during cold phase has been analysed for the TC value during this short interval of time as a specific case study. The calculated TC of (-7.4) over a range of 0.75 °C has been appropriately presented at its temperature stimulus. This evaluation of TC value is different from the regular evaluation of TC value which is performed with a 2°C difference as mentioned in materials and methods part. Subsequently after this brief cold phase, the neuron does not show any activity. The neuron again exhibits activity only after a thorough washout with normal ACSF. During the recovery sinus, the neuron remains cold-sensitive in nature. This kind of high cold-sensitivity where the neuron has not been blocked under calcium free ACSF is occasional. Only one such neuron could be blocked under calcium free ACSF (Nakashima *et al.,* 1987). Cold-sensitive neurons were also revealed in another whole-cell study (Kobayashi and Takahashi, 1993) and few PO/AH neurons retaining their cold sensitivity during synaptic blockade (Hori *et al.,* 1980). On the other hand a cold-sensitive neuron which has been totally blocked during the superfusion with calcium free ACSF has been shown in (figure 15). In this case, the neuron exhibits cold sensitivity in both the control sinuses (normal ACSF). During synaptic blockage, the neuron is totally suppressed and no firing rate has been exhibited. Subsequently TC value during synaptic blockage could not be evaluated. After washout, a recovery sinus is made which retains its cold sensitivity. This is a case of synaptically induced cold-sensitive neuron.

However, it is doubtful if Ca^{++} free ACSF removes all influences of presynaptic sensory neurons, where Ca^{++}- independent but voltage-dependent secretion occurs (Parnas *et al.,* 2000; Yang *et al.,* 2005). In view of these observed aspects, the present investigations were carried out with the previous experiences of cold sensitivity observed in different bird species and also in the chicken which exhibited inherent tendency. This could also be substantiated where the change in the neuronal activity and thermosensitivity in the blocking medium is not necessarily underlain by the block of synaptic inputs into a cell. Replacement of calcium with magnesium in the neurons themselves may differently affect the excitability of these neurons. Schmid and Pierau (1993) found the dependence of firing rate and thermosensitivity of PO/AH neurons on the calcium ion concentration and concluded that replacement of calcium with magnesium to distinguish between inherent and synaptically driven neuronal thermosensitivites is not applicable. This condition might be applied when blockade-induced changes in neuronal activity and thermosensitivity occur. However, it is clear that in cases when both neuronal firing rate and thermosensitivity are not changed by a synaptic blocking medium they are the intrinsic properties of the neuron. Invariably in the present study the existence of such inherently cold sensitive neurons in some populations of PO/AH neurons have been shown (figure 14).

Additionally, in the current series of experiments, five cold-sensitive neurons were also tested for inherent nature under synaptic blockage and under the action of GABA receptor agonists also. In a total of 5 cold-sensitive neurons, one exhibited inherent tendency and 4 were blocked under the action of $GABA_A$ receptor agonist muscimol. Similarly 2 cold-sensitive neurons showed inherent tendency and were not blocked under the synaptic action and also under the action of $GABA_B$ receptor agonist baclofen. This might also indicate that the cold-sensitive neurons during synaptic block and simultaneously under the action of their respective GABA receptors might exhibit inherent tendency (figures 16 a, b and c). In the (figure 16 a), initially the neuron was found to be cold-sensitive under the synaptic block. Later the neuron was subjected to synaptic block with calcium free ASCF and also to the action of $GABA_A$ receptor agonist muscimol simultaneously. At this stage the neuron was tested under calcium free ACSF and also under muscimol during the sinus (figure 16 b). The neuron which was suppressed totally during the start of the sinus and also in warm phase, exhibited activity under the cold phase and was found to cold-sensitive. The same neuron was given a long wash and stimulation was given during the normal ACSF where it maintained its cold sensitivity. After this, the

neuron was subjected to synaptic block and also to the action of $GABA_B$ receptor agonist baclofen (figure 16 c). The neuron was again suppressed during the warm phase and showed activity in cold phase, thus exhibiting cold sensitivity. Similarly after a long wash it showed recovery under normal ACSF. This kind of sensitivity might also reflect the inherent tendency of the neurons when a synaptic block with calcium free ACSF was performed simultaneously under the action of their respective GABA receptor agonists wherein the neurons exhibited activity. *In vivo* studies suggest the involvement of calcium in thermoregulation (Myers *et al.,* 1976). The extracellular calcium has been shown to be a physiological parameter that changes during the nerve cell activity. Mechanical stimulation of cat's paw, for instance decreases the extracellular calcium in the cortex from 1.3 mM to 0.7mM (Heinamann *et al.,* 1977). The use of low calcium and high magnesium solution to block synaptic transmission takes advantage of the well established dependence of neurotransmitter release on the extracellular calcium concentration (Katz and Miledi 1970; Kuno and Takahashi 1986). In view of the present preliminary investigations, additional studies with specific receptor blockage are necessary to shed light on further identification of primary cold-sensitive neurons in juvenile birds.

The high cold sensitivity could be proposed in relation to the biological phenomena which occur in the early postnatal life of the precocial birds.

As already mentioned under the sub heading of the discussion under 'Neuronal hypothalamic thermosensitivity: early postnatal developmental profile in chicken', a generally higher hypothalamic cold sensitivity was found in juvenile precocial birds. Furthermore changes within hypothalamic thermosensitivity within the prenatal period did always affect the proportion of cold-sensitive neurons in a stronger way which suggests a possible thermoregulatory role of cold-sensitive PO/AH neurons in prenatal and juvenile birds. Another plausible proposition to explain this high cold sensitivity in hypothalamic thermosensitivity in precocial birds within the perinatal period might come from the fact that in contrast to mammalian embryos precocial bird embryos and also the young chicken and Muscovy ducklings develop independently of their parents and might therefore be affected much more often by a decrease in ambient temperature (due to the parents leaving the egg). Although avian embryos are very tolerant to this drop in ambient temperature nevertheless temperature might have an influence on the development of neuronal hypothalamic thermosensitivity during the early post natal periods.

And there exists evidence which comes from the observations that in contrast to autonomic thermoregulatory mechanisms in avian embryos thermoregulatory behaviour is

developed at a very early stage. Acoustic responses can be used in some bird species for protection of endothermic reactions and therefore cold induced vocalisations are signals of the offspring's in need for warmth. Close relationships between cooling and calling rate have been observed (Nichelmann and Tzschentke 1997). In Geese immediately after immersing the egg in cold, the distress call rate of the embryo increases because of the cold stimulus and after re-warming this rate decreases again (Nichelmann and Tzschentke 2003). These observations are in agreement with results of experiments carried out by Evans *et al.* (1994) in ring-billed gulls (*Larus delawarensis*). In this case, embryos vocalised strongly when body temperature dropped below 36°C. The network is morphologically formed in the PO/AH region during the first 3-5 postnatal weeks (Gerocs *et al.,* 1986).

Cold-sensitive as well as warm-sensitive neurons, integrating information on skin and hypothalamic temperature, might receive inputs from peripheral thermoreceptors (Boulant, 1974; Boulant *et al.,* 1989) within early development. Even though this might not lead to an effective activation of heat loss and heat retention mechanisms, the incoming cold-signals from peripheral thermoreceptors might have an influence on the development of hypothalamic thermosensitivity. Temperature stimuli are know to alter the activity of warm-sensitive and cold-sensitive neurons and similar fluctuations in the extracellular calcium levels may occur in the PO/AH after thermal challenge (Schmid and Pierau 1993). And as such the two types of neuronal cold sensitivity *viz.,* synaptically driven cold sensitivity in some units and inherent neuronal cold transduction in other units, contribute to the general cold induced excitation of cold-sensitive PO/AH neurons, leading to activation of heat production in response to cooling.

In conclusion, the high neuronal hypothalamic cold sensitivity seems to be a specific characteristic feature of the early development in birds and possibly in mammals. A species specificity of the early development of neuronal hypothalamic thermosensitivity in birds could be clearly demonstrated. Primarily inherent cold-sensitive neurons probably exist in the birds PO/AH, which could act as thermosensors.

4.2 Modulatory action of GABAergic substances during thermoregulation

In the present study a total of 160 neurons were investigated under different conditions with GABAergic substances to determine the effect of $GABA_A$ and $GABA_B$ receptor agonists and antagonists. GABA exerts its effects by acting at two pharmacologically distinct receptors, the $GABA_A$ receptor and $GABA_B$ receptor. In birds the GABAergic

involvement in the control of body temperature might occur via the control of the activity of temperature sensitive and temperature insensitive PO/AH neurons. The present investigations were conducted in two series of experiments which involves the studies conducted with baclofen (35 neurons) in the first series. In the second series of experiments a comprehensive study with both $GABA_A$ and $GABA_B$ agonists and antagonists was made with 125 neurons. In the same series cold-sensitive neurons were studies under synaptic blockade as well as previously discussed under the sub heading 'Thermoregulatory relevance of high hypothalamic cold sensitivity in precocial bird species'.

4.2.1 Effect of $GABA_B$ agonist on different kinds of neurons with special reference to cold-sensitive neurons: Series 1

In the ensuing studies the effect of $GABA_B$ receptor agonist in the postnatal period has been investigated in series 1. In the initial investigations the influence of $GABA_B$ agonist baclofen was tested in 35 neurons. Among these 13 neurons were insensitive (37%), 11 warm-sensitive (31.5%) and 11 cold-sensitive (31.5%) which responded with alteration of tonic activity (firing rate) and temperature coefficient also as shown in figure 17. There are different investigations to explain the neuronal mechanisms of $GABA_B$ receptors and its agonist baclofen.

In comparison with warm-sensitive and temperature insensitive neurons, cold-sensitive neurons show very clear reactions under pharmacological influence. On the whole the present data demonstrates that the majority of neurons (97%) in the PO/AH of chicks respond to the $GABA_B$ receptor agonist. High number of cold-sensitive PO/AH neurons respond to the $GABA_B$ receptor agonist baclofen, indicating that these neurons express functional $GABA_B$ receptors. This was also proved in the subsequent experiments where majority of neurons (97%) in the PO/AH of chicken respond to $GABA_B$ receptor agonist. Also in the rat PO/AH a high percentage (97%) of neurons responded to baclofen (Yakimova *et al.,* 1996). For the whole population of investigated cold-sensitive neurons a significant decrease in firing rate and a significant increase in TC was found in the present study. In warm-sensitive and temperature insensitive neurons the influence on firing rate was lower and no significant changes in the TC were observed (Yakimova 2005). As shown in figure 18, the effect of $GABA_B$ receptor agonist baclofen on a cold-sensitive neuron has been assessed where the firing rate was inhibited. Recovery in firing rate and temperature sensitivity was observed after washout of the substance which shows the modulatory action of baclofen.

Studies on the effects of GABAergic agents on temperature sensitive neurons in rat hypothalamus have shown that the temperature sensitivity of rat PO/AH neurons is only changed by ligands of $GABA_B$ receptors and this effect has been restricted to temperature-sensitive neurons. While the tonic activity was decreased, the TC was significantly increased by $GABA_B$ agonist baclofen (Yakimova *et al.,* 1996). Related to the influence of baclofen on TC, the present results are in concurrence with these findings. The TC either increased or decreased in a number of neurons. In juvenile Muscovy ducks after bombesin application, similar results were found (Tzschentke *et al.*, 2000) but this result was in contrast to that observed in adult rats, where the main reaction on bombesin was an increase in the TC (Schimd *et al.,* 1993). These differences of changes in TC after $GABA_B$ agonist as well as bombesin application between juvenile and adult organisms may be related to the peculiarity of early development of body functions, as first changes in physiological parameters on endogenous and exogenous influences are nonspecific. During later development a qualitative change occurs, hence these changes become specific (Tzschtentke and Basta, 2000, 2002; and Tzschtentke *et al.,* 2004). No relationship between changes in firing rate and TC after $GABA_B$ agonist application was found in the present study. This is similar with observations, for instance, after bombesin application in juvenile Muscovy ducklings (Tzschtentke and Basta, 2000) or TRH superfusion in the adult rat (Tzschtentke *et al.,* 1994). However, the increase in trends in TC among the temperature sensitive neurons was also found in chickens but clear trends were especially established in cold-sensitive neurons, which are noted to be in relatively high percentage in the PO/AH of juvenile chicks. While only about 3% of rat PO/AH neurons were cold-sensitive (Yakimova *et al.,* 1996), 31.5% of chicken PO/AH neurons were determined as cold-sensitive by the present investigations. A similar high neuronal cold sensitivity in the PO/AH was also found in embryonic and juvenile Muscovy ducks (Tzschentke and Basta 2000) as already discussed under the sub heading 'Neuronal hypothalamic thermosensitivity: early postnatal developmental profile in the chicken'.

There are different investigations to explain the neuronal mechanisms of $GABA_B$ receptors and its agonist baclofen. On postsynaptic neurons the $GABA_B$ receptor mediated increase in K^+ conductance generates fast and slow inhibitory post synaptic potentials (IPSPs). This mechanism can lead to a hyper polarization often observed in neurons of different brain areas and is probably the main reason for the inhibition of neuronal activity. It has been demonstrated that IPSPs, most probably of GABAergic origin, can increase the interspike interval and that this ability is augmented by cooling, i.e. temperature sensitivity

can be modulated by inhibitory synaptic input (Curras *et al.,* 1991). Consequently, the promotion of GABAergic postsynaptic potentials by $GABA_B$ agonist could increase temperature sensitivity of hypothalamic neurons. On the other hand, $GABA_B$ receptors may act at presynaptic sites by altering the release of neurotransmitters such as norepinephrine, dopamine or 5-HT via presynaptic mechanisms (Clark and Lipton 1985). And also the hypothalamic neurons endogenously express to a varying degree for an after hyperpolarization, an inward rectification with an inward current and K^+ current. Such intrinsic and transmitter activated conductance likely serve as important determinants of firing patterns of hypothalamic neurons. In the hypothalamic slices of guinea pigs, with the bath application of $GABA_B$ receptor agonist baclofen the hypothalamic neurons respond with a membrane hyperpolarization or an outward current. And as such majority of the neurons tested with $GABA_B$ receptor agonist baclofen responded with membrane hyper polarization or an outward current. The terminals of both inhibitory (GABAergic) and excitatory (glutamergic) afferents are generally subjected to modulation by presynaptic $GABA_B$ receptors and that this modulation is potentially directed to inhibitory inputs and these observations on the role of $GABA_B$ receptors in modulating postsynaptic inhibitory action prove this aspect (Kolaj *et al.,* 2000). In addition, slow GABAergic IPSPs might facilitate discharge frequency by removal of the inactivation of the low-voltage activated Ca^{2+} currents (Crunelli and Leresche 1991); however it is not known whether this facilitation is modulated by temperature changes. On the other hand shortening of action potentials and decrease of after-hyper polarization with increasing temperatures appear to contribute to neuronal temperature sensitivity (Pierau, Klee and Klussmann 1976; Curras *et al.,* 1991). The shortening of action potentials due to an inhibition of Ca^{2+} currents and the subsequent reduction of after-hyper polarization due to a reduced Ca^{2+} activated K^+ current by $GABA_B$ receptor activation observed by several investigators (Matsushima, Tegner, Hill and Grillner 1993) might promote temperature sensitivity. Although the cellular mechanisms of the temperature sensitivity of hypothalamic neurons is not completely understood, intracellular recordings suggest that action potentials of warm-sensitive neurons are preceded by pre-potentials or pacemaker potentials and that the rate of rise of these depolarizing potentials increases with warming (Curras *et. al.,* 1991). Modulation of potentials could be a possible mechanism by which $GABA_B$ receptor activation changes temperature sensitivity.

Jha *et al.*, (2001) clearly suggest that normally GABA exerts a direct inhibitory action on cold-sensitive neurons, while it acts on presynaptic heteroreceptors, possibly on

the norepinephrinergic afferent input terminals of the warm-sensitive neurons, for mediating its action

Presynaptic inhibition is a form of neuromodulation that interacts with activity dependent plasticity. The apparent affinity of baclofen is strongly reduced during physiologically relevant stimulus and also the overall inhibition experienced by a synapse. This has been clearly substantiated where $GABA_B$ mediated presynaptic inhibition is more accurately described as a high pass filter than as simple inhibition, which can be used to assess the effects of presynaptic inhibition under physiologically relevant conditions (Ohliger-Frerking *et al.,* 2003). There are differences in the pharmacodynamics of $GABA_B$ receptor activation in between GABAergic neurons from the hypothalamic regions. And the levels of synaptically released GABA for instance from the terminals of the suprachiasmatic neurons can influence the relative contribution of pre versus postsynaptic $GABA_B$ receptors in the modulation under excitatory and inhibitory neuronal innervation to parvocellular neurons (PVN).

As already discussed the present data indicates that no direct correlation exists between the changes in TC and changes in firing rate induced by $GABA_B$ receptor agonist, suggesting that different mechanisms might be involved in the modulation of neuronal temperature sensitivity and tonic activity. This notion is in accordance with the results upon $GABA_B$ receptor mediated changes of the temperature sensitivity of rat PO/AH neurons, demonstrating a complete divergence of the drug effect on TC and tonic activity (Yakimova *et al.,* 1996).

Nevertheless, inhibition of firing rate and the specific changes of temperature sensitivity of temperature sensitive PO/AH neurons observed after application of the $GABA_B$ receptor agonist baclofen may be attributed to the role of GABA mediated activity of PO/AH neurons which play an important role in temperature regulation in birds. The present work in chicken elucidates a comparative analysis on the influence of baclofen in temperature sensitive neurons where earlier works were limited to mammals.

In the second series of experiments a further a comprehensive investigation was carried out in relation to both the agonist and antagonist of GABAergic substances.

4.2.2 A comprehensive study on the plasticity of hypothalamic neurons: effect of GABAergic substances: Series 2

In the present study, a systematic examination of chicken brain slices with respect to temperature sensitivity and GABAergic neuronal activity was initiated. The modulation of GABAergic process is assessed through their specific agonists and antagonists. So this question was addressed by examining the effects of GABA on neuronal firing in PO/AH, a region that is vital to the control of body temperature. Synaptic plasticity was studied under a combination of$GABA_A$ and $GABA_B$ agonists and antagonists. This study was necessary in view of the previous investigations with $GABA_B$ receptor agonist baclofen to substantiate the comprehensive action of different GABAergic substances. In this subsequent investigation 125 PO/AH chick neurons were studied with GABAergic substances in different combinations as shown in tables 9 and 10. In the actual study in chicken main effects of GABAergic substances on firing rate and TC of PO/AH neurons are similar to mammals. $GABA_A$ receptor agonist muscimol as well as the $GABA_B$-receptor agonist baclofen significantly inhibited the tonic activity of the hypothalamic neurons *viz.,* paraventricular nucleus (PVN), regardless of their type of thermosensitivity (figures 19 and 22). In contrast, the $GABA_A$ receptor antagonist bicuculline, and also the $GABA_B$ receptor antagonist CGP 35348 increased firing rate of the majority of the neurons (figures 19 and 22). The results show that the chick PO/AH neurons, which are possibly involved in thermoregulation, express functional $GABA_A$ and $GABA_B$ receptors. These findings are in agreement with observations in mammalian PO/AH neurons, especially the rat's hypothalamus, where the tonic activity of the majority of temperature sensitive and insensitive neurons was inhibited by muscimol and baclofen. Application of the respective antagonists show the opposite effect (Yakimova *et al*., 1996). The neuronal network in the anterior hypothalamus appears to be under tonic control of continuously released GABA, since the $GABA_A$ antagonist bicuculline and $GABA_B$ antagonist CGP35348 (although in higher concentration) increase the spontaneous activity of warm and cold-sensitive as well as insensitive neurons. Indication that the GABA effect might be receptor specific came from the experiments of Serrano *et al.* (1985), which demonstrated that the GABA induced hypothermia is not blocked by bicuculline, suggesting that the GABA effect on thermoregulation is not primarily mediated through activation of $GABA_A$ receptors. Since intraperitoneal, as well as intraventricular, application of baclofen in doses between 5-10 mg kg-' (i.P.) and 5-15 ng (i.c.v.) induced hypothermia in mice, it was suggested that $GABA_B$ receptor stimulation is important for the hypothermic effect of GABA (Gray,

Goodwin, Heal & Green, 1987; Jackson & Nutt, 1991). However, $GABA_A$ receptors may also participate in thermoregulation since pretreatment with the $GABA_A$ antagonist bicuculline (3 mg kg-1 i.P.) augments the hypothermia induced by low doses of baclofen (1-10 mg kg-1 i.P.) in restrained rats (Sancribian *et al.,* 1991). The antagonistic interaction of $GABA_A$ and $GABA_B$ receptors in thermoregulatory responses might be due to a modulating effect of $GABA_A$ on $GABA_B$ receptors as is indicated by the augmentation of the baclofen induced increase of temperature sensitivity of cold-sensitive hypothalamic neurons in the presented experiments.

Effect of different concentrations of GABAergic substances

Interaction of $GABA_B$ receptors with other receptor mechanisms may also play a role in GABA mediated changes of body temperature; e.g. higher doses of baclofen (30 mg kg-1 i.P.) produced an initial temperature fall (30 min) but then increased body temperature, probably due to activation of opioid and/or prostaglandin mediated mechanisms (Sancribian *et al.,* 1991). An increase in body temperature due to activation of brown adipose tissue was also seen after intraventricular administration of baclofen in anaesthetized rats (Horton, LeFeuvre, Rothwell & Stock, 1988). This hyperthermia was abolished by simultaneous application of the $GABA_A$ agonist muscimol, again indicating $GABA_A$ and $GABA_B$ receptor interaction.

In the present experiments high doses of antagonists (10 times higher concentration) have been successfully used to block the agonist induced inhibition as shown in figures 24 and 25 b. The relatively high doses of antagonists are necessary to block the agonist induced inhibition has also been reported by others (Kerr, Ong, Prager, Gynther and Curtis 1987). The morphological substrates for this tonic inhibition are GABA containing small and medium sized neurons that have been demonstrated in different areas of the hypothalamus (Otterson and Storm Mathisen 1984; Decavel and Van den Pol 1990). Furthermore, spontaneous GABA release could be demonstrated in the PO/AH *in vivo* (Jarry, Perschel and Wutke 1988). Intracellular recordings have revealed frequent spontaneous inhibitory post synaptic potentials (IPSPs) in warm and cold-sensitive hypothalamic neurons (Curras, Kelso and Boulant 1991) and these IPSPs are most probably mediated by GABA (Hoffman, Wuarin and Dudek 1994). The small spikes recruited during bicuculline superfusion in some of the neurons also indicate that the spontaneous activity of a number of neurons is inhibited via GABAergic mechanism.

GABA exerts its effects by acting at two pharmacologically distinct receptors, the $GABA_A$ and $GABA_B$ receptor (Kerr & Ong, 1995**;** Johnston, 1996). Whereas $GABA_B$ receptor is coupled with G-protein and negatively linked to adenylyl cyclase, the $GABA_A$ receptor exerts its action, it being an ion channel (ligand-gated chloride ion channel superfamily). Both may have different effects on the sensitivity of the cells to stimulation by cAMP-dependent mechanisms (Buzzi *et al*., 1997).

Action of baclofen in relation to other GABAergic substances

In the present study in chicks as discussed previously in relation to $GABA_B$ receptor agonist baclofen as well in studies carried out in mammals only the $GABA_B$ substances significantly change the temperature sensitivity in one special type of thermosensitive PO/AH neurons. Following the assumption, that the main thermoregulatory effect of neurotransmitters, neuropeptides and other substances is to modulate thermosensitivity (increase or decrease in TC) (Pierau & Schmid, 1990; Yakimova *et al*., 1996), the findings in both chicken as well as mammals are in agreement with the earlier notion that the GABA effect on temperature regulation is preferentially mediated by $GABA_B$ receptors (Serrano, 1985; Jackson & Nutt, 1991). Recent study in mammals investigating the effect of baclofen on PO/AH neurons by whole-cell patch clamp recordings are in accordance with these findings. The decrease in firing rate during baclofen application was accompanied with statistically significant membrane hyperpolarization in PO/AH neurons, as well as the resistance test investigated were significantly decreased during baclofen superfusion. The increase in TC among the warm-sensitive neurons was found (Yakimova, 2006). Activation of $GABA_B$ receptors by GABA or baclofen, mediated by a G-protein, causes a decrease in Ca^{2+}-condactance (Wu & Saggau, 1995) or an activation of a potential Ca^{2+}-dependent K^+-conductance (Bowery, 1993). $GABA_B$ receptors can be located either presynaptically or postsynaptically (Bettler *et al*, 2004). Presence of $GABA_B$ receptors in the mammalian PVN were shown by morphological studies (Bischoff *et al*., 1999). Electrophysiological studies indicate that in the PVN, functional $GABA_B$ receptors were found on axon terminals of GABAergic presynaptic neurons contacting magnocellular neurons (Cui *et al*., 2000) as well as parvocellular neurons (Liu *et al*., 2006). In rat brain slices application of the $GABA_B$ agonist baclofen decreased the frequency of spontaneous and miniature inhibitory postsynaptic currents (i.p.s.c.) and also the amplitude of evoked i.p.s.c. Both effects were suppressed by the $GABA_B$ receptor antagonist CGP55845A. The results suggest that under physiological conditions, presynaptic $GABA_B$ receptors exert a tonic inhibition on GABA release (Liu *et al*., 2006). Similar inhibitory control of GABA,

acting on $GABA_A$ receptors, via co-activation of $GABA_B$ receptors was also found in early development. During the prenatal brain development an important interaction between the excitatory $GABA_A$ and the substantial inhibitory action of $GABA_B$ receptors was found (Obrietan & Van den Pol, 1998). $GABA_B$ receptors could play a role as autoreceptors providing negative feedback control for synaptic GABA secretion (Anderson and Mitchell1985; Pittaluga *et al*., 1987).

During these investigations no significant changes were observed in TC values during co-application of substances. This shows that the neuronal activity is stabilized during the application of the GABAergic substances. This is evident from the neuronal stabilization when $GABA_A$ agonist muscimol (figure 20) and its antagonist bicuculline (figure 21) were applied in different kinds of neurons. This is an evidence for specific $GABA_A$ as well as $GABA_B$ mechanisms in the modulation of tonic activity and thermosensitivity of hypothalamic neurons in birds. The inhibition of the influence of $GABA_B$ agonist baclofen on tonic activity and thermosensitivity in the brain slices of birds by application of the $GABA_B$ receptor antagonist CGP 35348 could be based on similar mechanism like in mammals as described before. The neuronal network in the anterior hypothalamus appears to be under tonic control of continuously released GABA, since the $GABA_A$ antagonist bicuculline, as well as the $GABA_B$ antagonist CGP 35348 influence the spontaneous activity and/or temperature sensitivity of chick PO/AH neurons.

Model of GABAergic action in thermoregulation in chicken in relation to other species

The present studies in chicken try to elucidate whether the different effects of GABAergic substances on hypothalamic neurons correspond to the changes in temperature regulation observed. In mammals, PO/AH neurons can be affected by different neurotransmitters and neuromodulators (Pierau *et al*., 1998). In avian species experiments on plasticity of neuronal hypothalamic thermosensitivity are scarce. In brain slices of juvenile birds the modulatory effect of the neuropeptide bombesin (Tzschentke *et al*., 2000) and of prenatal epigenetic temperature adaptation (Tzschentke & Basta, 2002) on hypothalamic thermosensitive and insensitive neurons was shown. The actual study shows, that GABAergic substances can also modulate thermosensitivity of hypothalamic neurons and might play a role in body temperature control in birds like observed in mammals. Microinjection into the avian midbrain shows that the GABAergic system is involved in the control of sleep and temperature homeostasis (Yekimova & Pastukhov, 2002).

Similar to the observations made in mammals GABA is a major inhibitory neurotransmitter in the central nervous system of birds also (Veenman & Reiner, 1994; Sun *et al*., 2005). In mammals GABAergic receptors are functional very early in the hypothalamic development providing most of the initial excitatory drive and are expressed widely in the hypothalamus (Decavel & Van den Pol, 1990, Represa & Ben-Ari, 2005).

Earlier studies have shown that various neurotransmitters and neuromodulators which affect temperature regulation by intrahypothalamic or intraventricular application may change the activity of PO/AH neurons (Cabanac, Stolwijk & Hardy, 1968; Eisenman, 1969; Boulant, 1980; Hori, 1991). Under *in vivo* as well as *in vitro* conditions it was observed that substances causing hypothermia, such as bombesin and capsaicin, usually enhance tonic activity of warm-sensitive PO/AH neurons (Hori, Shibata, Kiyohara, Nakashima & Asami, 1988; Schmid, Jansky & Pierau, 1993) while those leading to hyperthermia, such as pyrogens, interferon-z, prostaglandin E2, have a depressing effect (Cabanac *et al.,* 1968; Eisenman, 1969; Nakashima, Hori, Kuriyama & Matsuda, 1988). Spontaneous activity of temperature-insensitive neurons was also enhanced by the hypothermic substances bombesin and capsaicin but was little or not affected by hyperthermic substances. This is in agreement with neuronal models of hypothalamic control of body temperature which predict that an increased activity of warm-sensitive neurons causes hypothermia by activating heat loss mechanisms, while the reduction of firing rate of warm-sensitive neurons activates mechanisms for heat conservation and heat production (Bligh, 1981). However, the observed decrease of spontaneous activity of warm-sensitive hypothalamic neurons after application of GABA agonists does not fit to the model which would predict that substances causing hypothermia increase the tonic activity in rats. Some experiments have revealed that the effect on spontaneous activity might not be the main indicator for a specific action of a substance on PO/AH neurons. For example, bombesin and substance P both increase the spontaneous activity of temperature-sensitive hypothalamic neurons (Schmid *et al.,* 1993) but only bombesin considerably decreases body temperature by intrahypothalamic application (Jansky, Riedel, Simon, Simon-Oppermann and Vybiral, 1987) while substance P either has no effect or causes a small increase of body temperature. What differentiates the two types of substances is the effect on the temperature sensitivity of PO/AH neurons. Bombesin increases the TC of the majority of warm-sensitive neurons and of almost all temperature-insensitive neurons. The effect on the latter leads to a recruitment of warm-sensitive neurons resulting in an increase in the signal output of the warm pathway to the effector neurons. Substance P on the other

hand decreases the temperature sensitivity of most of the warm-sensitive neurons but has very little effect on the TC of temperature insensitive neurons. The hypothesis was put forward that a substance which decreases body temperature increases the temperature sensitivity of PO/AH neurons while a decrease of the TC is characteristic for hyperthermic substances. This thesis has been confirmed for other substances affecting thermoregulation (Pierau *et al.,* 1994). The $GABA_B$ substances fulfill the criteria of the above hypothesis since the temperature sensitivity of warm-sensitive PO/AH neurons is significantly increased by the $GABA_B$ agonist baclofen and decreased by the $GABA_B$ antagonist phaclofen in rats. But in chicken this kind of action is seen in cold-sensitive neurons only and this might be another argument for the role of cold-sensitive neurons in thermoregulation in chicken and in birds as well. However, in contrast to bombesin, the TC of temperature-insensitive neurons was not significantly changed, although there was a tendency for a decrease, and consequently no recruitment of warm-sensitive neurons was obtained by baclofen in rats. The present results showing that only $GABA_B$ substances significantly change the temperature sensitivity of cold-sensitive neurons is in agreement with the notion that the GABA effect on temperature regulation is preferentially mediated by $GABA_B$ receptors (Serrano *et al.,* 1985; Jackson & Nutt, 1991 and Yakimova *et al.,* 1996). The observation that the $GABA_A$ antagonist bicuculline augments the increase in the temperature senisitivity of hypothalamic neurons induced by the $GABA_B$ agonist baclofen offers a possible explanation for the amplification of the baclofen-induced hypothermia by the $GABA_A$ antagonist bicuculline observed in restrained rats (Sancibrian *et al.,* 1991).

Overview of GABAergic action in chicken

The presented results support the hypothesis that changes in temperature sensitivity of hypothalamic neurons rather than an effect on their tonic activity are connected to the hypo- or hyperthermic action of a substance. The action of a drug on spontaneous activity of PO/AH neurons is, however, also likely to contribute to its effect on body temperature. Presently it remains unresolved as to how the two parameters interact. In addition, the interaction of pre and postsynaptic $GABA_A$ and $GABA_B$ pathways, as well as the possible activation of other mediators such as opioids or prostaglandins by GABAergic substances, suggests a high degree of complexity in the neuronal network involved in temperature regulation.

Thus it is clearly evident from the action of the GABAergic substances that they have varied effects on different kinds of temperature sensitive and insensitive neurons. The action of GABAergic substances stabilizes the neuron under most of the circumstances as observed in different figures. These changes lead to neuronal plasticity in the chicken hypothalamic neurons.

Thus, the main effects of GABA, mediated via the $GABA_A$ and $GABA_B$ receptors on thermosensitive and insensitive PO/AH neurons in the chicks are similar with that described in mammals with certain differences. This is a clear indication that this mechanism is highly conserved during evolution of the amniotes. The present investigations enunciate main difference in respect of the $GABA_B$ receptor mediated change in hypothalamic neuronal temperature sensitivity. In chicken this action was restricted to cold-sensitive neurons, whereas in mammals this effect was only seen in the warm-sensitive neurons. We could arrive at this conclusion only after making a comprehensive investigation of $GABA_A$ receptor and $GABA_B$ receptor agonists and as well as their antagonists in chicken hypothalamic neurons.

5 CONCLUSIONS

From the present study it is evident that the neuronal hypothalamic thermosensitivity is characterized by high plasticity in chicken. A typical developmental pattern has been found in chicken in the present studies. The thermosensitivity during the characterization has shown two different levels exhibiting two different stages of development in the postnatal period. Thus this work reveals two phases of development where, in the first phase neuronal cold sensitivity shows a gradual increase and warm sensitivity shows a decline (until 20 days age group). And there occurs a second stage from 20 days to 30 days old age group wherein the cold sensitivity decreases and warm sensitivity augments. In the process of development at a later period cold sensitivity might cease to have its impact. A similar kind of developmental pattern was observed in Muscovy duck (Tzschentke and Basta 2002) but the differentiation in thermosensitivity occurs at a different time period. Thus a qualitative change might occur from the "juvenile" to the "adult" type in bird species which may be species specific.

Another major aspect of the present study is in relation to the possible thermoregulatory role of cold-sensitive neurons. The high neuronal cold sensitivity seems to be a specific characteristic feature of early development in birds, which has been one of the focal aspects of the present work. The present investigations suggest that primarily inherent cold-sensitive neurons probably exist in addition to the synaptically induced cold-sensitive neurons in the PO/AH of birds, which could act as thermosensors.

The neuronal network in the anterior PO/AH appears to be under the tonic control of continuously released GABA since the $GABA_A$ antagonist bicuculline as well as $GABA_B$ antagonist CGP35348 influence the spontaneous activity and temperature sensitivity in chicken. In the present investigations, an increasing trend was found in temperature coefficient (TC) of warm-sensitive neurons but a significant increase in TC was established in cold-sensitive neurons. The main effects of GABA, mediated via the $GABA_A$ and $GABA_B$ receptors on thermosensitive and insensitive PO/AH neurons in the chicks are similar with that described in mammals. The main difference has been established in respect of the $GABA_B$ receptor mediated change in hypothalamic neuronal thermosensitivity. In chicken this action was restricted to cold-sensitive neurons, whereas in mammals this effect was only seen in warm-sensitive neurons.

The future perspective lies in a more detailed analysis of hypothalamic thermosensitivity as well as cold sensitivity in relation to GABAergic substances. The outcome of the present study forms a platform to understand the developmental status in

chicken in the early postnatal life. But additional investigations are necessary to look into the neuronal thermosensitivity in adult birds which might reveal the direction of further plasticity in the bird species. To facilitate a comparative analysis with the mammalian species, studies are required in relation to the embryonic and the juvenile forms in mammalian species.

Especially studies in the direction of membrane polarization could shed light on the complexity of neuronal network involved in thermoregulation during the early postnatal life and also during adult periods in birds and mammals. This would enable to understand if the cold sensitivity is inherent or synaptically induced. The GABAegic studies in relation to excitatory postsynaptic currents and inhibitory postsynaptic currents using whole cell-patch clamp technique are necessary in this relation.

To conclude the present research in birds elucidates a comparative analysis on the influence of GABAergic substances on temperature sensitive and insensitive neurons, in addition to the studies on plasticity of neuronal hypothalamic thermosensitivity. This is a step in filling up this lacuna in birds where earlier works were confined to mammals.

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PUBLICATIONS

1. **Nagaraja Sallagundala**, Krassimira Yakimova and Barbara Tzschentke (2006) Characterization of neuronal hypothalamic plasticity in chicken: a comparative analysis. World Poultry Science Association Journal (In press).

2. K.Yakimova, **N.Sallagundla** and B.Tzschentke (2005). Influence of Baclofen on Temperature–Sensitive Neurons in Chick Hypothalamus. Methods Find ***Exp Clin Pharmacol*** 2005, 27 (6): 401-404.

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SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.
Desweiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsverordnung. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrades.

Berlin, den……

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Sallagundala Nagaraja